THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

VOLUME 149
BALTIMORE
1943

PUBLISHED AT YALE UNIVERSITY FOR
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, INC.
BALTIMORE, U. S. A.

CONTENTS OF VOLUME 149

No. 1, July, 1943

Letters to the Editors

No. 2, AUGUST, 1943

Letters to the Editors

HURAIN, A NEW PLANT PROTEASE FROM HURA CREPITANS

By WERNER G. JAFFÉ

(From the Department of Chemistry, Instituto Quimio-Biologico, Caracas-Los Rosales, Venezuela)

(Received for publication, March 15, 1943)

A considerable number of publications exist which describe plant proteases, but our knowledge of the chemistry of these enzymes and their mode of action is scant. Except for the ferments of the group of papainases, which have been relatively well investigated, there are few experimental data on other plant proteases, so that classification outside of this group is, as yet, impossible. In the present paper some experimental data obtained with a new plant protease are reported which may be helpful for the purpose of classification.

Botanical Data—The new ferment was isolated from the sap of the tree *Hura crepitans* (commonly known in Venezuela as *jabillo*) of the family of Euphorbiaceae. Proteases so far have been identified in three members of this family, *Croton tiglium*, *Ricinus communis*, and in the sap of *Euphorbia palustris* (1).

EXPERIMENTAL

Isolation—When the bark and roots of *Hura crepitans* are cut, a brown, turbid, and caustic sap appears (which the natives use to remove bad teeth). This sap is slightly acid and has a pH of about 5 to 5.5. Sap isolated during the dry season yields 20 per cent of residue on drying; during the rainy season, about 17 per cent. It contains no caoutchouc. When centrifuged, the insoluble matter separates slowly and the supernatant liquid becomes clear; this, added to twice its volume of acetone, forms a white precipitate. The weight of the dried precipitate is 10.2 per cent that of the original sap. The precipitate was purified by re-solution and precipitation in the same manner. The resulting white powder is a protein and proteolytic enzyme herewith named "hurain." All the experiments were made with this crude preparation.

The insoluble matter, separated by centrifuging, was washed with water and recentrifuged until the wash water did not form a precipitate with acetone. The residue is a gray powder; its yield is 3 per cent of the crude sap. It may be further purified by solution in 1 N sodium hydroxide, filtration, and precipitation with acid. This purified residue shows the same proteolytic activity as hurain, and has been named "hurain *i*" (insoluble).

Chemical Properties—Hurain in aqueous solution is precipitated by satu-

ration with ammonium sulfate or sodium chloride; this precipitation is more complete if the solution is acid or alkaline. When alcohol is slowly added to an aqueous solution of hurain, precipitation begins when the alcohol concentration reaches about 30 per cent, and is completed when it reaches about 90 per cent. From acid solution, alcohol precipitates only part of the protein; the remaining fraction may be precipitated from the alcohol solution by neutralization. This fraction is also soluble in strong alkaline alcoholic solution.

Hurain gives positive Millon, xanthoproteic, and biuret reactions. Mineral acids, picric acid, mercuric chloride, and 0.1 M silver nitrate precipitate it. Sodium nitroprusside gives no color reaction with hurain. The iodine-azide reaction of Feigl for SH groups is positive, but this reaction is often positive for S—S groups too (2). When heated, hurain begins to coagulate at 90° and coagulates rapidly at 100°.

The viscosity of a hurain solution depends upon the pH. In slightly acid solution it has a low viscosity. When very slightly alkaline, it rapidly becomes so highly viscous that it is comparable to a pectin solution. In strongly alkaline solution the viscosity decreases again. In 5 M urea this change of viscosity is not observed. Similar observations have been made with gelatin solutions. In the latter case, too, urea inhibits the increase of viscosity which is observed normally when the solution is cooled (3). The hurain fraction which is soluble in acidified alcohol does not show this change of viscosity.

Isoelectric Point—An attempt was made to determine approximately the isoelectric point of hurain by means of a method (4) based on the fact that invert soaps precipitate only the anions of proteins (5). A concentrated solution of the ferment was mixed with different buffer solutions (McIlvaine's buffer) and a few drops of a 1:1000 invert soap solution (dimethylalkylbenzylammonium chloride) were added. The protein was precipitated between pH 4.25 and 4.6. The isoelectric point therefore probably lies between pH 4 and 5.

Hurain i—The water-insoluble hurain *i* is soluble in 1 N sodium hydroxide and in a borate buffer of pH 9. When acidified, it precipitates immediately from these solutions, but cannot be precipitated by boiling or by saturation with ammonium sulfate. It is also soluble in acid alcohol, and is precipitated by neutralization. It also dissolves in strong urea solution, and precipitates when the solution is diluted.

Hurain *i* is probably a secondary product. While the sap from the bark and roots of the tree *Hura crepitans* is turbid, the sap obtained from leaves and buds is clear and colorless but readily forms a white precipitate which has the same characteristics as hurain *i*. A similar product is obtained from hurain which has been kept for some time in strong alkaline solution.

Proteolytic Properties.—To study the proteolytic properties, 10 per cent ferment solutions or the filtered sap (which also contained 10 per cent ferment) was used. To 0.25 ml. of these solutions, 0.25 ml. of activator or inhibitor solution, respectively, was added. The mixtures were kept at laboratory temperature (27°) for 24 hours. Then 9.5 ml. of the substrate solution were added and the whole mixture incubated at 37° for 6 hours. After this, formol titration was performed. As substrates, 2 per cent gelatin solution and 2 per cent peptone solution (Witte's peptone), which contained 10 per cent of 0.2 M dibasic sodium phosphate to adjust the pH to 8, were used.

Results

Table I shows a comparison of the activities of hurain and ficin, both as natural saps which had been adjusted to contain 10 per cent of ferment.

TABLE I

Comparison of Activities of Hurain, Hurain i, and Ficin

The values give the quantity of 0.1 N NaOH employed in the formol titration.

Ferment	Substrate	0.1 N NaOH
		ml.
Ficus sap	Gelatin	2.15
" "	Peptone	0.65
Hura "	Gelatin	1.80
" "	Peptone	1.59
Hurain, 10% solution	Gelatin	1.66
" 10% "	Peptone	1.52
" *i*, 10% suspension	Gelatin	1.40
" " 10% "	Peptone	1.31

The *Ficus* sap used was a commercial product. It had the same activity as fresh papaya latex in 1:1 dilution. It appears that the gelatin-splitting activity of hurain is somewhat lower than that of the ficin, while the peptone-splitting activity is higher. Activation of the *Ficus* sap upon peptone could not be produced to any measurable degree.

Table II shows some results of investigations which were carried out in order to establish the activating and inhibiting action of a number of chemicals. It is evident that activators and inhibitors of papainases are without effect. Only four substances were found to influence markedly the activity of hurain, silver nitrate, mercuric chloride, iodine, and nitrous acid. Greenberg and Winnick (6) have pointed out that these same substances inhibit the activity of solanain, a proteolytic enzyme from *Solanum elaeagnifolium*. Nitrous acid was found by Philpot and Small (7) to reduce the activity of pepsin by 50 per cent. A similar reduction has been proved for solanain

and hurain. These three ferments give a yellow color reaction with the reagent.

Fig. 1 summarizes experiments to establish the optimum pH for gelatin-splitting of hurain. It was found to be approximately 8.

Milk-Clotting Activity—For the clotting of milk by hurain, the simple relation $E \cdot t = K$ (where E is the quantity of ferment, t the time necessary to clot a constant quantity of milk at a given temperature, and K the constant of the process), which has been shown to express the clotting process

TABLE II

Effect of Some Activation and Inhibition Substances on Hurain

The values are expressed in per cent of the value obtained with the pure enzyme.

Substance		Gelatin digestion	Peptone digestion
	M		
Cysteine	0.1	98	101
Hydrocyanic acid	0.1	102	106
Hydrogen peroxide	0.1	100	98
Maleic acid .	0.1	97	96
Phenylhydrazine	0 1	103	98
Iodine	0.1	7	3
Silver nitrate	0.02	8	18
Mercuric chloride	0.02	3	5
Nitrous acid ..	0.1	65	73

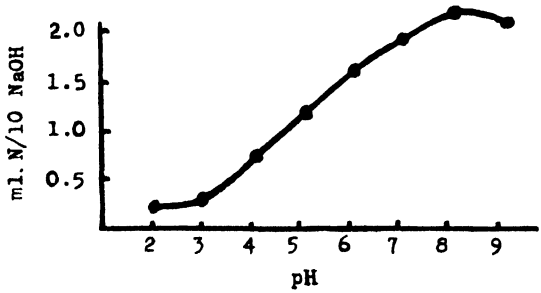


FIG. 1. Hydrolysis of gelatin mixtures, pH 2 to 9, by hurain

of chymotrypsin, applies, while the formula derived by Balls and Hoover (8) for papain does not.

The milk-clotting power of hurain is very low compared with its gelatin- and peptone-digesting activity. Fresh hurain has 5 milk-clotting units per gm. as defined by Balls and Hoover, if 5 ml. of milk are used at a temperature of 30°, and 30 units per gm. at 40°. For activated papain the corresponding values are 200 units per gm. at 30° and 300 units per gm. at 40°. Curve 1 in Fig. 2 expresses graphically the milk-clotting process of hurain and Curve 2 that of papain.

Action on Intestinal Parasites—Intestinal parasites are not attacked by

the proteolytic enzymes of the intestinal tract, owing to an antiferment which nullifies the action of these ferments and protects the parasites from their action. It has been demonstrated in recent years that papainases are not inhibited in their activity by these antiferments and therefore can digest living *Ascaris* and other intestinal parasites (9).

The action of hurain on *Ascaris* was determined by the following experiments. Three test-tubes were filled with 15 ml. of crude, unfiltered *Hura* sap and a fourth with crude *Ficus* sap. In the first tube were placed three living specimens of *Ascaris lumbricoides* from the human body; in the second, three dead worms of the same species; and in the third, three living earthworms. Three living *Ascaris* were put in the tube with the *Ficus* sap. All tubes were incubated at 37° for 18 hours. After this time the earthworms were dead and showed superficial ulcerations, while only one *Ascaris* had died and all appeared unattacked. The *Ascaris* in *Ficus* sap

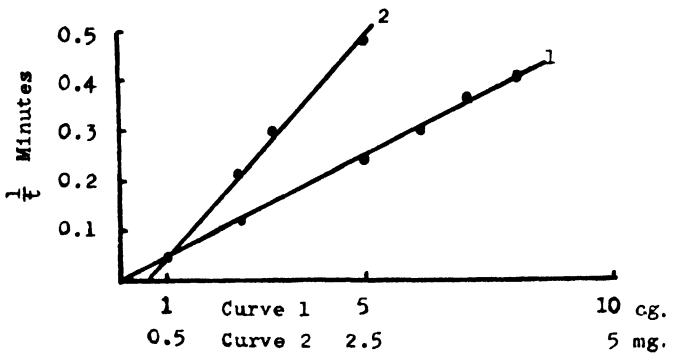


FIG. 2. Relationship between clotting time (reciprocal) and amount of enzyme. Curve 1, hurain; Curve 2, activated papain.

were strongly attacked and partly digested. The tubes were allowed to stand for 2 days more. By then, the earthworms were almost completely digested, forming a slimy mass, while all *Ascaris* appeared unattacked, but dead. The *Ascaris* in *Ficus* sap were as completely digested as the earthworms. It is evident from these experiments that hurain does not digest living or dead *Ascaris*, but digests earthworms. This difference certainly is due to the antiferments, characteristic of intestinal parasites like *Ascaris*.

Toxicity—Richet (10) studied the toxicity of the sap from *Hura crepitans*. He found that 0.1 ml. of the crude sap kills a rabbit within half a minute and that the toxic dose for dogs is 1 mg. of the crude protein derived from the sap per 100 kilos of body weight. This minimal dose killed the dogs within 3 days. He attributed this toxic effect to a toxalbumin which he named crepitin.

To check the toxicity, ten mice were given orally 0.5 ml. of the filtered *Hura* sap by means of a stomach tube. All animals survived the adminis-

tration for more than 8 days. To ten other mice was given 1 ml. each. These animals died within 24 hours. Stomach and intestines were filled with a slimy brown liquid. Anatomical lesions could not be detected. These results are not conclusive for oral toxicity, because death may be caused in mice by 1 ml. doses of solutions which are osmotically active or not neutral (11).

Of ten mice injected intraperitoneally with 0.1 ml. of the filtered sap, eight died within 24 hours, while of ten injected in the same way with 0.1 ml. of the sap diluted 1:1, only one animal died within 24 hours. There were no anatomical lesions visible. In the course of 1 week the two remaining animals of the first group and four more of the second group died. All had inflammation and necrosis in the peritoneum. All mice used in these experiments weighed between 16 and 18 gm.

Four rabbits were given intravenous injections of 1 ml. of the filtered *Hura* sap. All the animals survived the observation period of 4 days. Two rabbits were injected intraperitoneally with 5 ml. of the sap. These also survived 4 days, but all the animals of both groups died within 2 weeks. All the rabbits employed weighed between 1.5 and 1.7 kilos. The sap used in these experiments was collected in July. A sample obtained from the same tree in December was twice as toxic.

As toxalbumins derived from Euphorbiaceae have the property of agglutinating red blood cells, the *Hura* sap was checked with respect to this activity. It was found to agglutinate rat blood cells in a dilution of 1:100-000. Washed cells from human blood are agglutinated by the sap diluted 1:10 but not by a dilution of 1:100. No hemolytic action could be detected.

There exists a marked discrepancy between the toxicity of *Hura* sap found by Richet in Brazil and by the author in Venezuela. In both cases, however, a retarded action of doses insufficient to cause an acute toxic effect has been established. It has been shown that the toxic and the blood-agglutinating principles of ricin are not identical (12). If this is also true for crepitin, it is possible that the toxic factor is absent from the sap of *Hura* of Venezuela and that the retarded toxicity is due to a distinct factor. If the toxic effect is due to the toxalbumin crepitin of Richet, it must be present in a concentration lower than 1^{-6} gm. per ml. of sap.

DISCUSSION

The most important characteristics of hurain may be summarized as follows: chemical behavior like that of an albumin; maximum of activity in alkaline range; no inhibition by merely oxidizing agents like hydrogen peroxide; positive Philpot and Small reaction; inability to digest intestinal parasites. These properties distinguish it clearly from the papainases. In the incomplete literature available to the author there is no description

of a plant protease with such properties. Nevertheless, it seems probable that among the great number of vegetable proteolytic enzymes, mentioned in the literature without detailed description, there are some which may possess the above characteristics. It is very probable that solanain recently studied by Greenberg and Winnick (6) may belong to the same group as hurain, because it shows a positive Philpot and Small reaction and has its maximum of activity at pH 8.5. It may be worth while to check the similarity of some other plant proteases to hurain. Perhaps a new group of these ferments may be established similar to the group of the papainases.

The papainases strongly resemble in their activity the catheptic ferments of animal origin. Vegetable ferments which are supposed to have analogous relations to pepsin have been found in *Drosera rotundifolia* (13). In the case of hurain a relation to trypsin may be suggested. All the characteristics summarized above are also true for trypsin. The reaction of Philpot and Small was checked for trypsin and found to be similar to that of hurain. Other characteristic properties of trypsin, especially the activation by enterokinase and by Mg^{++}, are as yet unchecked for hurain. As it is the purpose of this publication to describe some properties of the new enzyme which were easy and rapid to establish in order to find some eventual relationship to other plant ferments, no attempt to purify the crude product was made, and therefore these properties have not yet been checked for hurain.

SUMMARY

A new proteolytic enzyme, named hurain, has been isolated from the tree *Hura crepitans*. Some of the properties which distinguish it clearly from the papainases are described. A possible relationship to trypsin is discussed.

BIBLIOGRAPHY

1. Wehmer, C., Die Pflanzenstoffe, Jena, **2**, 674, 696 (1931).
2. Feigl, F., Qualitative analysis by spot test, Amsterdam, 184 (1937).
3. Signer, R., and Mosimann, H., *Helv. chim. acta*, **24**, 1058 (1941).
4. Jaffé, W. G., *J. Biol. Chem.*, **148**, 185 (1943)
5. Kuhn, R., and Bielig, H. J., *Ber. chem. Ges.*, **73**, 1080 (1940).
6. Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **135**, 761 (1940).
7. Philpot, J. S. L., and Small, P. A., *Biochem. J.*, **32**, 542 (1938).
8. Balls, A. K., and Hoover, S. R., *J. Biol. Chem.*, **121**, 737 (1937).
9. Berger, J., and Asenjo, C. F., *Science*, **91**, 387 (1940).
10. Richet, C., *Compt. rend. Soc. biol.*, **66**, 663 (1909); *Chem. Abst.*, **4**, 595 (1910); *Ann. Inst. Pasteur*, **23**, 745 (1909); *Chem. Zentr.*, **1**, 1033 (1910).
11. Martin, G. J., Fisher, C. V., and Thompson, M. R., *Arch. Int. Med.*, **69**, 662 (1942).
12. Fuchs, L., and Falkensammer, H., *Sc. pharm.*, **10**, 103 (1939).
13. Holter, H., and Linderstrom-Lang, K., *Z. physiol. Chem.*, **214**, 223 (1933).

HISTAMINE AND PROTEOLYTIC ENZYMES

LIBERATION OF HISTAMINE BY PAPAIN

By M. ROCHA E SILVA AND SYLVIA O. ANDRADE

(From the Department of Biochemistry and Pharmacodynamics, Instituto Biologico, São Paulo, Brazil)

(Received for publication, March 31, 1943)

Previous papers have shown that trypsin is very active in releasing histamine from the living tissues (1, 2). This would suggest, as indicated before (3), that histamine is bound to cells forming peptide linkages with the amino acid chain of cell proteins. On the other hand, the fact that chymotrypsin is very ineffective in releasing the histamine,[1] would indicate that the linkages of this substance with the cell proteins display a certain specificity toward proteolytic ferments. Among the innumerable peptides tested by Bergmann and his associates (4, 5), only a few are split by trypsin: benzoyl-*l*-argininamide, benzoyl-*l*-lysinamide, hippuryl-*l*-argininamide, and hippuryl-*l*-lysinamide. Trypsin is a proteinase which attacks a peptide linkage involving the carboxyl group of a basic amino acid. Consequently, we might conclude that the linkage of histamine with the cell proteins is of the amide type, the next amino acid residue being either arginine or lysine. A further step presented in this report has been to fractionate papain and to show that its histamine-liberating activity at pH 7.3 to 7.5 runs parallel to its benzoyl-*l*-argininamide-splitting component.

The methods developed by Bergmann and his associates (6), which have been used in the following experiments, proved rather successful, despite the fact that the bindings of histamine with the cell constituents are labile and by no means realize the conditions of simple stable substrates, such as those used in the study of proteolytic enzymes. Furthermore, the amounts of histamine to be liberated are so small that one has to use a biological test to detect them; consequently, one has to compare results obtained with purely chemical methods with those obtained in biological experiments. The results presented in this paper, however, show that quite clear information can be gathered from the application of methods of enzymatic chemistry to the solution of problems of liberation of biologically active substances.

Material and Methods

The papain preparation used was obtained by purification of a crude dried latex, according to the procedure described by Irving, Fruton, and Bergmann (7). A stock solution of papain was prepared by dissolving

[1] Rocha e Silva, M., unpublished results.

200 mg. of purified papain in 25 cc. of saline. The protein nitrogen content of this preparation was 2.6 mg. per cc. From this solution, 2.5 cc. were transferred to a 5 cc. volumetric flask and 1 cc. of cysteine solution (0.5 M) + 1.5 cc. of phosphate (pH above 6) or citrate (pH below 6) buffer was added.

The enzymatic experiments were performed in 2.5 cc. volumetric flasks containing 0.5 to 1 cc. of the papain-cysteine solution, 1.25 mM of substrate,[2] and 0.3 cc. of the corresponding buffer solution. The final concentration of cysteine in the solution was 0.04 mM per cc. The flasks were incubated at 39° and samples of 0.2 cc. were taken at varying intervals for estimation of the free carboxyl groups by the Grassmann-Heyde method (8).

From each analysis the first order constant $K = 1/t \log a/a - x$ was determined and the average K of three or four determinations with the same substrate was used to calculate the respective proteolytic coefficient (C_{BAA}, C_{CIG}, C_{LA}, etc.). The value of C_X was calculated by dividing the average constant K by the concentration of the enzyme, expressed in mg. of protein nitrogen contained in 1 cc. of the solution (for more details, see (6)).

The histamine experiments were performed according to the method previously described (2). Into each of three or four flasks were put 1 cc. of papain solution and 1 cc. of saline. Samples of 7 to 8 cc. of heparinized rabbit blood, obtained by heart puncture, were added to each flask. After standing 5 to 10 minutes at room temperature and 3 minutes at 38°, the samples were centrifuged and 2 cc. of the supernatant plasma added to 4 cc. of trichloroacetic acid. A 2 cc. sample of total blood was also taken. The histamine extraction and estimation were performed according to Code's method (9).

Results

Tissue cathepsins and papain are complex mixtures of proteolytic enzymes which split innumerable peptides of the most varied type (10). To study the possibility of applying to the histamine problem the methods of enzymatic chemistry, we have chosen papain as a representative of this group of enzymes. As is well known, tissue cathepsins are almost inactive above pH 6. Papain, being a vegetable cathepsin and having many of the components of animal cathepsins, is still fairly active at pH 7 to 7.5. As shown in Fig. 1, papain-cysteine has two definite optima, one around pH 5 and the other around pH 6.8 to 7. At pH 7.3 to 7.5, papain-cysteine splits benzoyl-*l*-argininamide very quickly, carbobenzoxyisoglutamine,

[2] Most of the substrates used in the following experiments were prepared by one of us in Dr. M. Bergmann's laboratory at The Rockefeller Institute for Medical Research, New York.

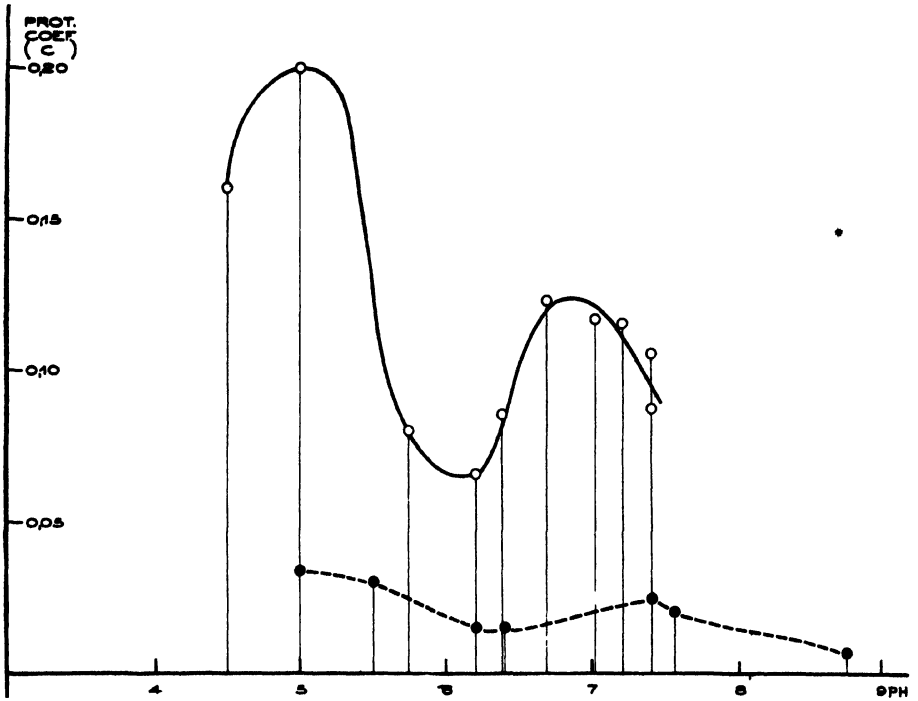


FIG. 1. Influence of pH on the splitting of benzoyl-*l*-argininamide (continuous line) and carbobenzoxyisoglutamine (dotted line) by papain-cysteine. At the ordinates the proteolytic coefficients are shown (C_{BAA} and C_{CIG}), and at the abscissas the pH.

TABLE I

Splitting of Different Substrates by Papain-Cysteine, at pH 7.3 to 7.5

Enzyme concentration, 0.13 mg. of protein N per cc.

Substrate	Time	Hydrolysis	Proteolytic coefficients ($\times 10^4$)
	min.	*per cent*	
Benzoyl-*l*-argininamide	30	62	
	40	77	
	120	91	$C_{BAA} = 1200$
Carbobenzoxyisoglutamine	80	29	
	120	40	
	150	49	$C_{CIG} = 140$
Hippurylamide	75	14	
	120	22	$C_{HA} = 70$
l-Leucinamide	155	6	
	275	12	$C_{LA} = 15$
Glycylglycine	110	1	
	140	3	$C_{GG} = 0.7$
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	1440	0	$C_{CGT} = 0$
Glycyl-*l*-leucine	1440	0	$C_{GL} = 0$
Carbobenzoxy-*l*-leucylglycine	1440	0	$C_{CLG} = 0$
Chloroacetyl-*l*-tyrosine	1440	0	$C_{CT} = 0$

hippurylamide, and *l*-leucinamide less quickly, and glycylglycine very slightly (Table I). Carbobenzoxy-*l*-glutamyl-*l*-tyrosine, carbobenzoxy-*l*-leucylglycine, and glycyl-*l*-leucine are not split by papain above pH 7.

TABLE II

Liberation of Histamine from Rabbit Blood Cells by Papain

Papain A was prepared directly from a fresh stock solution of papain. Papain B was prepared by treating 4 cc. of the stock solution of papain with 0.5 cc. of N NaOH. After 2 hours of contact, the solution was neutralized with N HCl and the cysteine solution and the phosphate buffer added. Papain C was prepared from a stock solution of papain which had been heated for 12 to 15 minutes at 52° or 2 hours at 39° (Experiment 8).

Experiment No.	C_{BAA}			Histamine content				
	Papain A	Papain B	Papain C	Plasma + saline	Plasma + Papain A	Plasma + Papain B	Plasma + Papain C	Total blood
				γ per cc.	γ per cc	γ per cc	γ per cc.	γ per cc.
1	0.11			0.50	1 15			1.25
2	0.11	0		1.10	2 20	1.15		2.00
3	0.12	0		1.00	2 00	1.20		2.00
4	0.11	0		0.42	2.00	0.40		0.42
5	0.12	0	0.12	0.25	0.90	0.27	0.90	1.00
6	0.10	0	0 10	0.30	1.40	0 60	1.30	0.94
7	0.12		0 09	0.25	0.50		0.50	0.75
8	0.12		0.10	0 25	0 50		0.50	0.75

TABLE III

Liberation of Histamine from Rabbit Blood Cells by Dialyzed Papain

Papain A was prepared from a fresh stock solution of papain. Papain B was prepared by dialyzing the solution of papain for 20 hours against distilled water in the ice box.

Experiment No.	C_{BAA}		Histamine content			
	Papain A	Papain B	Plasma + saline	Plasma + Papain A	Plasma + Papain B	Total blood
			γ per cc.	γ per cc.	γ per cc.	γ per cc.
1		0.11	0.20		0.75	0.62
2		0.11	0.10		0.75	
3	0.12		0.50	0.60		0.60
4	0.08	0.08	1.00	2.00	2.00	1.25
5	0.11	0.06*	0.42	2.00	0.80	1.20
6	0.10	0.10	0.85	1.20	1.15	1.40

* Dialyzed 48 hours in the ice box; the C_{BAA} of this solution of papain was abnormally low.

As the experiments on histamine liberation ought to be done at a pH range (7.3 to 7.5) in which the curve of activity of papain-cysteine begins to decline, a certain variation in the values of the proteolytic coefficient

C_{BAA} should be expected. This would explain small variations of the proteolytic coefficients as shown in Tables II and III.

The results presented in Tables II, III, and IV definitely show that the ability of papain to liberate histamine from rabbit blood cells runs parallel with the activity of its benzoyl-*l*-argininamide-splitting component. This component is fairly resistant to heat; as shown in Table II, heating papain at 52° for 12 to 15 minutes does not change the activity of the enzyme upon benzoyl-*l*-argininamide or its histamine-liberating capacity. On the

TABLE IV

Effect of Treatment of Papain with Isopropyl Alcohol on Histamine Liberation from Rabbit Blood Cells and Benzoyl-l-argininamide-Splitting Activity

Papain A was prepared directly from a stock solution of purified papain containing 200 mg. of papain per 25 cc. of saline. From this solution 2.5 cc. were transferred to a 5 cc. volumetric flask and 1 cc. of cysteine solution + 1.5 cc. of phosphate buffer was added. Papain B was prepared by treating 10 cc. of the stock solution of papain with 20 cc. of chilled isopropyl alcohol; after 15 minutes at 4°, the precipitate was collected by centrifugation and redissolved in saline. After two or three precipitations with isopropyl alcohol, the volume was made up to the volume of the original stock solution. From this solution, 2.5 cc. were transferred to a 5 cc. volumetric flask and 1 cc. of cysteine solution + 1.5 cc. of phosphate buffer was added. Temperature of the bath, 39°; final pH, 7.3 to 7.5.

Experiment No	C_{BAA}		Histamine content			
	Papain A	Papain B	Plasma + saline	Plasma + Papain A	Plasma + Papain B	Total blood
			γ per cc	γ per cc.	γ per cc	γ per cc
1	0 120	0 055	0.04	0 46	0.15	0.52
2	0 081	0 066	0 25	0.81	0.74	0.63
3	0.120	0 095	0.30	1 25	0 80	1.70
4	0.088	0.056	0 12	1.25	0 85	1 80
5	0 096	0.040	0.26	0 56	0.35	0.90
6	0 093	0.040	0.26	0.65	0 35	0.90
7	0.086	0.061	0.35	3.12	2 50	1 80
Averages ..	0 097	0.059	0.23	1.16	0.82	1.18

$C^{B}_{BAA}/C^{A}_{BAA} = 0.61.$ $\dfrac{\text{Histamine B}}{\text{Histamine A}} = \dfrac{0\ 82 - 0.23}{1.18 - 0.23} = 0.62.$

contrary, contact with N NaOH, for ½ to 1 hour, was enough to destroy the benzoyl-*l*-argininamide-splitting component and at the same time its histamine-liberating activity. Furthermore, we have verified that dialysis against distilled water does not interfere with the papain-cysteine component which splits benzoyl-*l*-argininamide, but definitely lowers the activity of papain toward *l*-leucinamide, the C_{LA} dropping from a value of 0.0015 to 0.0007. At the same time, the liberation of histamine by papain is not altered appreciably by dialysis against distilled water (Table III).

That the liberation of histamine does not run parallel with the activity of the papain component which splits carbobenzoxyisoglutamine is shown in Table V, since treatment with isopropyl alcohol does not change very conspicuously the activity of papain-cysteine toward carbobenzoxyisoglutamine. There is no correlation between the changes of the proteolytic coefficients toward benzoyl-*l*-argininamide (C_{BAA}) and carbobenzoxyisoglutamine (C_{CIG}). This shows that the papain component which splits the former at pH 7.3 to 7.5 is not identical with the component which splits the latter. As shown in Table V, the quotient $C^{A}_{BAA}/C^{A}_{CIG} = 6.3$ before

TABLE V

Proteolytic Coefficients of Papain-Cysteine towards l-Argininamide and Carbobenzoxyisoglutamine

Papain A was prepared from a fresh stock solution of papain, while Papain B was prepared after three precipitations of the same solution by isopropyl alcohol, as indicated in Table IV.

C_{BAA}		C_{CIG}	
Papain A	Papain B	Papain A	Papain B
0.096	0.040		
0.093	0.040	0 020	0.013
0.088	0.056	0.013	0 016
0.106	0.055	0.011	0.014
0.081	0 066	0.020	0.015
0.115	0.061	0.011	0.014
0.106	0 060	0.020	0.015
0.112	0.053	0.015	0.012
0.130	0.065		
0.086	0.061		
Averages 0.101	0.056	0.016	0.014

treatment with isopropyl alcohol; after three precipitations with isopropyl alcohol, the quotient $C^{B}_{BAA}/C^{B}_{CIG} = 4$.

The data presented in Table IV, however, show that treatment of papain with isopropyl alcohol lowers at the same rate (0.61 and 0.62) the activity of the enzyme both in splitting benzoyl-*l*-argininamide and in releasing the histamine from rabbit blood cells, this being strong suggestive evidence that both activities depend on the same papain component.

DISCUSSION

There are substantial proofs that the histamine linkages with the cell constituents can be ruptured by proteolytic enzymes which display the same specificity as trypsin. The possibility that in several cases the nor-

mal cellular cathepsins might be the agent of histamine liberation would induce one to study the histamine-liberating activity of these complex proteolytic enzymes. Unfortunately, animal cathepsins are almost inactive at the pH of blood, which makes them unsuitable for experiments on histamine liberation from living tissues. The study of a vegetable cathepsin like papain gave satisfactory results. Papain is a mixture of proteolytic enzymes which display specificities somewhat similar to those shown by animal cathepsins. Papain has a pH optimum around 5, and needs activators such as HCN, cysteine, ascorbic acid, etc., exactly as the usual animal cathepsins do. It has been possible to identify in papain a proteolytic component similar to Cathepsin II (which splits benzoyl-*l*-argininamide), another similar to Cathepsin III (which splits *l*-leucinamide), and another similar to Cathepsin V (which splits carbobenzoxyisoglutamine). Papain is unable to split glycyl-*l*-leucine, and under the conditions of our experiments it did split hippurylamide slightly and glycylglycine very slightly (at pH 7.3 to 7.5). The great advantage offered by papain over animal cathepsins is the fact that it still retains an appreciable part of its activity at pH 7.4. The splitting of benzoyl-*l*-argininamide at this pH is fairly rapid, while animal cathepsins prepared according to the method of Anson (11) lose their activity almost entirely when the pH of the medium is maintained above 6. In consequence, papain can be used as a cathepsin model when the experiments must be performed at pH 7.4, as in those with rabbit blood.

We have been able to show that the histamine-liberating activity of papain does not show any relationship to its capacity for splitting *l*-leucinamide, glycylglycine, glycyl-*l*-leucine, carbobenzoxy-*l*-glutamyl-*l*-tyrosine, or carbobenzoxy-*l*-leucylglycine. On the other hand we have shown that submission of papain to several treatments, such as dialysis against distilled water, heating to 52° for 15 minutes, and contact with N NaOH, destroys some of its proteolytic components and that its capacity for splitting benzoyl-*l*-argininamide disappears at the same rate as its histamine-liberating activity. This shows a definite parallelism between the activity of the enzyme in splitting this substrate and in liberating histamine from rabbit blood cells. Treatment of papain with isopropyl alcohol lowered its activity in splitting benzoyl-*l*-argininamide, but did not markedly alter its activity in splitting carbobenzoxyisoglutamine; the effect of this treatment upon the histamine-liberating activity of papain was of the same order of magnitude as its effect upon the capacity for splitting benzoyl-*l*-argininamide.

The problem presented in this paper, therefore, concerns the probable nature of the linkages holding histamine to the cell proteins. Besides the fact that the release of histamine runs definitely parallel to the activity of

papain in splitting benzoyl-*l*-argininamide, it is worthy of note that this substrate is a typical one for trypsin, which is one of the most active substances in releasing the histamine from living cells. Chymotrypsin, which does not split this substrate, does not release histamine in appreciable amounts.

Among the very few simple substrates which trypsin has been shown to split are hippuryl-*l*-lysinamide and benzoyl-*l*-lysinamide, which indicates that lysine, instead of arginine, might be the amino acid residue proximal to the peptide linkage ruptured by trypsin. In this sense, one might draw the provisional conclusion that histamine is bound either to arginine or to lysine in the amino acid chain of cell proteins. On the basis of this conclusion, one would expect that Cathepsin II would probably be the cellular cathepsin component responsible for the release of histamine in the event that an abnormal activation of cellular proteinases is the *primum movens* of a discharge of this pharmacologically active substance.

SUMMARY

Papain-cysteine shows two optima of activity, at pH 5 and pH 6.8 to 7, when tested on synthetic substrates like benzoyl-*l*-argininamide and carbobenzoxyisoglutamine.

At pH 7.3 to 7.5 papain-cysteine splits benzoyl-*l*-argininamide, carbobenzoxyisoglutamine, hippurylamide, *l*-leucinamide, and, very slightly, glycylglycine. The other substrates tested, namely carbobenzoxy-*l*-glutamyl-*l*-tyrosine, glycyl-*l*-leucine, carbobenzoxy-*l*-leucylglycine, and chloroacetyl-*l*-tyrosine, were not split by papain-cysteine in that range of pH.

Papain was found to release histamine from rabbit blood cells to plasma. Dialysis against distilled water and heating at 52° for 12 to 15 minutes did not appreciably alter this effect of papain upon rabbit blood histamine. Treatment with NaOH definitely reduced to zero the ability of papain to release histamine. Several precipitations with isopropyl alcohol markedly reduced this capacity.

The papain-cysteine component which splits benzoyl-*l*-argininamide was altered by the above treatment in a quite similar manner, which led to the conclusion that the histamine-liberating activity of papain runs parallel with its capacity for attacking the arginine-amide linkage. The components which split *l*-leucinamide and carbobenzoxyisoglutamine were altered differently by the same treatment.

A provisional conclusion is drawn that histamine is present in the cell, forming an amide type of linkage, the proximal amino acid being either arginine or lysine.

BIBLIOGRAPHY

1. Rocha e Silva, M., *Arch. exp. Path. u. Pharmakol.*, **194,** 335, 351 (1940); *Arq. Inst. biol.*, São Paulo, **12,** 155 (1941).
2. Dragstedt, C. A., and Rocha e Silva, M., *Proc. Soc. Exp. Biol. and Med.*, **47,** 420 (1941).
3. Rocha e Silva, M., *J. Pharmacol. and Exp. Therap.*, in press.
4. Bergmann, M., Fruton, J. S., and Pollock, H., *J. Biol. Chem.*, **127,** 643 (1939).
5. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130,** 81 (1939); **138,** 243 (1941).
6. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138,** 231 (1941).
7. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **139,** 569 (1941).
8. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183,** 32 (1929).
9. Code, C. F., *J. Physiol.*, **89,** 257 (1937).
10. Bergmann, M., and Fruton, J. S., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1,** 63 (1941).
11. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130,** 19 (1939).

TRANSAMINATION AND PROTEIN SYNTHESIS IN GERMINATING OAT SEEDLINGS*

By HARRY G. ALBAUM† and PHILIP P. COHEN

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

(Received for publication, April 12, 1943)

The discovery of the transamination reaction by Braunstein and Kritzmann (1) has led to the suggestion that this reaction is of major importance in protein metabolism in plant and animal tissues. Thus Virtanen and Laine (2) have postulated that in the leguminous plants the first amino acid synthesized is aspartic acid, which in turn participates in the synthesis of the remaining amino acids by transamination with the corresponding α-keto acids. Unfortunately, quantitative data for plant tissues in particular have been strikingly inadequate, not only for support of the more elaborate theories, but even for the mere demonstration that transamination occurs at an appreciable rate. On the whole the studies with plant tissues have suffered from a poor choice of material and experimental conditions, in addition to inadequate analytical methods. Thus Cedrangolo and Carandante (3) reported that aqueous extracts of different seeds and germinated seedlings were most active in transaminating the system, α-ketoglutaric acid plus aspartic acid. The rates observed, however, were extremely low and of questionable significance (4). The reverse reaction was not investigated. No transamination was observed to occur in *Chlorella* (4), but the experimental conditions employed were not optimum in view of the findings reported in this paper.

Kritzmann (5) has reported the preparation of transaminating enzymes from animal and plant sources, and has found their properties to be similar. She has maintained the view that two transaminating systems exist, one concerned with glutamic (or α-ketoglutaric) acid, and the other with aspartic (or oxalacetic) acid. The latter system is reported by Kritzmann to be most readily prepared, free of the glutamic acid system, by extracting coarsely ground pea seedlings. Previous studies, however, by one of us (6–9) have led to the view that a single enzyme system is involved, the chief substrates for which are glutamic acid *plus* oxalacetic acid.

In this paper experiments are reported in which the reaction

(1) *l*(+)-Glutamic acid + oxalacetic acid $\underset{b}{\overset{a}{\rightleftharpoons}}$ α-ketoglutaric acid + *l*(−)-aspartic acid

* This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

† National Research Council Fellow in the Natural Sciences, 1942-43.

was quantitatively investigated in embryos from oat seedlings at various stages of germination. Simultaneous estimations of different nitrogen fractions of the embryos are also reported for the purpose of correlating transamination with protein metabolism.

Procedure

Preparation of Oat Embryos—Oat embryos used in the studies were prepared in the following way. Grains of *Avena sativa* L. var. Victland were hulled, soaked in distilled water for 2 hours in the light, and germinated in the dark at room temperature for 24 hours. After germination, the grains were planted in beakers in contact with moist filter paper, as previously described (10), to which distilled water had been added. The plants were then allowed to continue their growth in the dark. Immediately after the initial soaking period and at 24 hour intervals thereafter groups of seedlings were removed from the beakers and the embryos were carefully dissected from the endosperms.

Preparation of Homogenates and Incubation Mixtures—Groups of embryos varying in number between ten and 100, depending on the age, were homogenized in ice-cold 0.1 M phosphate buffer with a stainless steel homogenizer. In most of the experiments the pH was checked and when necessary adjusted to pH 8.0 with 1 M sodium bicarbonate. In other experiments the desired pH was attained by using a phosphate buffer of approximately the desired pH and then adjusting as before.

1 ml. samples of the homogenate were pipetted into test-tubes or 50 ml. flasks to which, in the case of the complete system (Reaction 1, *a*), the following additions were made, 0.3 ml. of 0.1 M glutamate and 0.3 ml. of 0.1 M oxalacetate (or corresponding amounts of α-ketoglutarate and aspartate in the case of Reaction 1, *b*). The glutamate and oxalacetate were adjusted to the proper pH before use. The oxalacetate was added to the system after the homogenate plus glutamate was shaken for 10 minutes at 38° to allow for temperature equilibration. The substrate concentration was 0.0187 M. The reaction was stopped, at the end of varying periods of incubation with continuous shaking, by the addition of 1.0 ml. of 10 per cent sodium tungstate and 1.0 ml. of 10 per cent sulfuric acid. 2.4 ml. of water were added to make a final volume of 6.0 ml., and the mixture filtered.

Analytical Procedures—Aspartic acid formation was determined by the chloramine-T method previously described (7). Determinations were usually carried out in duplicate. For the estimation of α-ketoglutaric acid, the method of Krebs was employed (11).

Total and soluble (non-protein) nitrogen was routinely determined on all homogenates and filtrates by a micro-Kjeldahl method in the usual way.

Results

Comparison of Rate of Reaction 1, a by Aspartic Acid and α-Ketoglutaric Acid Determinations—The rates of Reaction 1, *a* as measured both by aspartic acid and α-ketoglutaric acid formation with the same incubation mixture are shown in Table I. While the rates of transamination are lower than those shown in Fig. 2, which were obtained at pH 8.0, which is closer to the optimum pH of 8.6, the values obtained by the two methods show good agreement. The significance of the data in Table I is that, first, the two independent analytical methods yield the same results,

TABLE I

Rate of Transamination As Measured by Aspartic Acid and α-Ketoglutaric Acid Formation in Same Incubation Mixture

2 hour-old embryos; pH 7.4. The per cent transamination was calculated on the basis of 672 microliters of added glutamic acid.

Incubation time	Aspartic acid formation					α-Ketoglutaric acid formation				
	CO_2 evolved		Glutamic acid blank	Aspartic acid formed	Transamination	O_2 uptake	Blank, O_2 uptake	ΔO_2 uptake	α-Ketoglutaric acid	Transamination
		Average								
min	*microliters*	*microliters*	*microliters CO_2*	*microliters*	*per cent*	*microliters*	*microliters*	*microliters*	*microliters*	*per cent*
15	744 754	749	615	134	19 9	116.5	44	72 5	145	21.5
30	835 858	847	615	232	34 5	166 5	44	122 5	245	36.5
60	942 915	929	615	314	46 7	190 5	44	146 5	293	43.7
120	985 955 1020	986	615	371	55.2	222	44	178	356	53.1

and second, that the course of Reaction 1, *a* is as depicted, and thus the measured rates in this system are not due to, nor influenced by, any secondary reactions which would serve either to utilize the substrates, glutamic and oxalacetic acids on the one hand, or the reaction products, aspartic and α-ketoglutaric acids, on the other hand.

Effect of pH on Reaction 1, a—The effect of pH on Reaction 1, *a* (with embryos from grains soaked for 2 hours) is shown graphically in Fig. 1. It is seen that the optimum pH is at 8.6, with a relatively rapid decline in activity with both increasing and decreasing pH. This is somewhat in contrast to animal transaminase which shows an optimum activity at pH 7.5 (8), with a relatively slower decrease in activity with change in pH. Whether the optimum pH for these plant homogenates would obtain

for more purified preparations, it is not possible to predict. On the other hand, Kritzmann (5) has reported that the optimum pH for aqueous extracts of pea seedlings is the same as that for animal preparations; *viz.*, 7.4.

Preliminary experiments in this study were carried out at pH 7.4 because of Kritzmann's findings (5) and those reported for animal transaminase (8). However, as a result of the data shown in Fig. 1, all subsequent experiments were conducted at pH 8.0. The optimum pH was not employed, because it seemed desirable to use a phosphate buffer.

Rates of Reactions 1, a and 1, b—The rates of Reactions 1, *a* and 1, *b* are shown graphically in Fig. 2. It is seen that Reaction 1, *a* proceeds at a relatively rapid rate initially and approaches more slowly an equilibrium

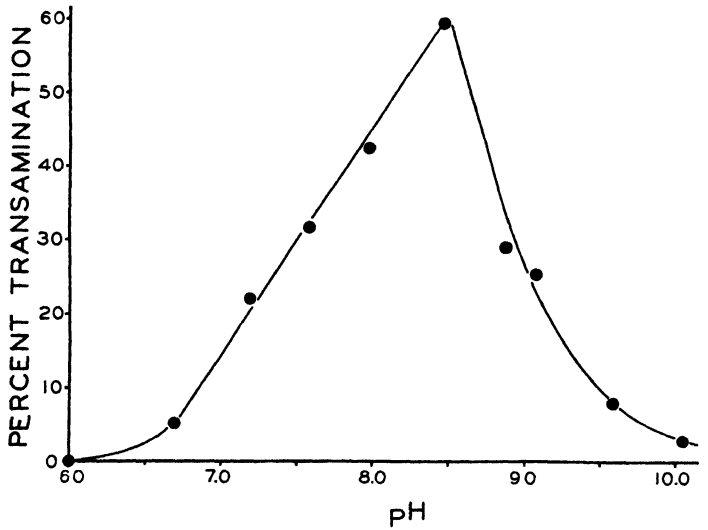


FIG. 1. pH-activity curve (Reaction 1, *a*). 2 hour-old embryos

level after about 60 per cent transamination. Reaction 1, *b* reveals a much slower initial rate, the equilibrium level being reached after about 20 per cent transamination. It is apparent that Reaction 1, *a* proceeds at a rate about 3 times as fast as Reaction 1, *b*. Values of this same order have been reported for purified transaminase from animal sources (8) and animal tissue homogenates (9).

Changes in Transaminase Activity (Units) and Protein and Non-Protein Nitrogen at Different Stages of Embryo Development—The units of transaminase activity present at different stages of embryo development are shown in Fig. 3. One transaminase unit is defined as that amount of enzyme which will produce 10 microliters of aspartic acid (or α-ketoglutaric acid) from a 0.0187 M concentration of glutamic acid plus oxalacetic acid in 15

minutes at pH 8.0 and 38°. In addition, protein nitrogen (total nitrogen minus soluble nitrogen) and soluble nitrogen (75 per cent of which is

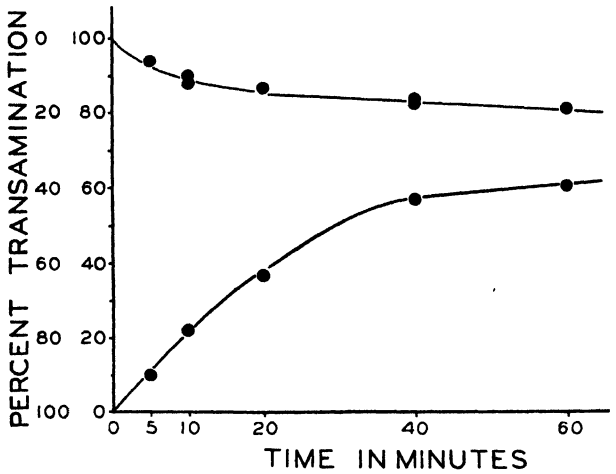


FIG. 2. Rates of Reactions 1, *a* and 1, *b*. 2 hour-old embryos; pH 8

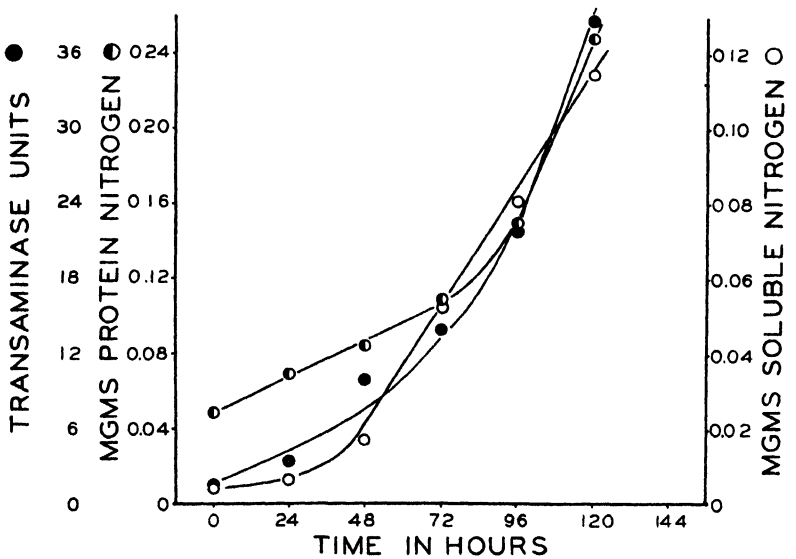


FIG. 3. Changes in transaminase activity and protein and non-protein nitrogen at different stages of embryo development. pH 8; Reaction 1, *a*.

amino nitrogen by the chloramine-T method) levels at the same stages are presented.

It can be seen from Fig. 3 that during the first 24 hours there is a slow and parallel increase in transaminase units, protein nitrogen, and soluble nitrogen levels. Between 24 and 48 hours, the soluble (or amino) nitrogen levels increase sharply. At about 48 hours, the transaminase units increase sharply. However, the protein nitrogen continues to increase at the same slow rate as during the first 24 hours up until the 72 hour stage. At this time the protein nitrogen levels rise sharply. Thus at 72 hours the transaminase units and soluble (or amino) nitrogen levels have reached relatively high values, subsequent to which time the protein nitrogen starts to increase rapidly. It would thus appear that the rate of protein synthesis is accelerated when the transaminase activity and the soluble (or amino) nitrogen values reach a certain level. It should be emphasized that, between 24 and 72 hours, the rate of synthesis of transaminase is more rapid than the rate of synthesis of total protein. This in itself suggests that there is a preferential synthesis of a key protein, transaminase, which either directly or indirectly influences the rate of total protein synthesis. After 72 hours the rates of transaminase synthesis and protein synthesis are parallel, again emphasizing the intimate association of these two constituents. It is at this 72 hour stage that other enzyme systems start appearing or enzyme systems already present increase markedly in activity (12, 13).

It is of interest to note that after 72 hours the soluble (or amino) nitrogen levels continue to rise along with the protein nitrogen level. It might be expected that if the protein were being synthesized from the amino acids (soluble nitrogen) present the latter values would decrease. During these early stages, however, the soluble (amino) nitrogen keeps increasing because of the rapid rate of transfer of soluble nitrogen from the endosperm which rapidly loses its protein nitrogen and supplies it to the embryo (12). Thus we have the conversion of the endosperm protein to the embryo protein through the medium of soluble (amino) nitrogen.

The function of transaminase thus might appear to be that of rearranging or interconverting the soluble (amino) nitrogen provided by the endosperm into the components necessary for synthesis of embryo proteins. The broader implications of this will be considered under the discussion.

$Q_{transamination}$ Values at Different Developmental Stages—A comparative measure of transaminase activity is conveniently expressed in terms of the conventional $Q_{transamination}$ values. However, since these values are usually expressed on the basis of the dry weight of the tissue, erroneous values are obtained with plants owing to the increasing amounts of supporting tissue, such as cellulose, with development. A more satisfactory basis for expressing $Q_{transamination}$ values is on the basis of mg. of protein, since the latter more adequately reflects the metabolically active tissue of the plant. With this in mind, $Q_{transamination}$ values are presented in Table II both on a dry

weight basis (Q_T) and on a protein basis ($Q_{T(Pr.)}$) at different stages of embryo development. It is apparent from Table II that Q_T values decrease with embryo development, while the $Q_{T(Pr.)}$ values increase. The latter values undoubtedly are a better reflection of the changes in transaminase activity than the former.

The $Q_{T(Pr.)}$ values are remarkably high and indicate that at least in this plant tissue transaminase activity is not only high initially but actually increases about 4-fold after 120 hours. During the first 24 hours the values change very little, but between 24 and 72 hours the values are tripled, while between 96 and 120 hours they are quadrupled.

The $Q_{T(Pr.)}$ value for Reaction 1, *b* in 2 hour-old seedlings is of the order of 100. Calculation of the $Q_{T(Pr.)}$ values for the same reaction in extracts from pea and corn seedlings from the data of Cedrangolo and Carandante (3, 4) gives values of 1.1 and 3.8 respectively.

TABLE II

Change in Q_T and $Q_{T(Pr.)}$ with Time in Developing Oat Embryos

$$Q_T = \frac{\text{microliters substrate transaminated}}{\text{mg. dry weight} \times \text{hrs.}}.$$

$$Q_{T(Pr.)} = \frac{\text{microliters substrate transaminated}}{\text{mg. protein} \times \text{hrs.}}.$$

Time	Q_T	$Q_{T(Pr.)}$
hrs.		
2	109	268
24	158	295
48	154	784
72	94	805
96	73	905

The $Q_{T(Pr.)}$ values for plant tissue are more or less comparable with Q_T values for animal tissue. In a comparison of the transaminase activity of animal and plant tissues it appears that in both cases there is a progressive increase with age (14). However, the values for the oat seedling are several times greater than those reported with the most active animal tissues. Thus pigeon breast muscle has a Q_T value of 450. On the other hand, purified transaminase preparations from pig heart muscle have values of the order of 1650 (8).

DISCUSSION

The exact manner in which transamination can function in synthetic reactions of protein is as yet not clear. Two possibilities merit consideration. In the first place, if one considers protein synthesis to proceed by the reversed action of proteolytic enzymes, then it is difficult to see how

transamination can tie up directly with this process. On this basis one must assume that transamination serves the function of rearranging or interconverting the amino acid substrates in such a way as to insure the proper concentrations and kinds of amino acids. The two amino acids, glutamic and aspartic (and their corresponding amides and α-keto acids), are known to be of special importance in intermediary protein metabolism in plants (15, 16). Unfortunately little is known about the influence of individual or combinations of different amino acids on the synthetic reactions of protein. In the second place, the possibility exists that protein synthesis is not a simple reversal of proteolysis.

The synthesis of peptides by condensation of glyoxals with amino compounds, followed by dehydrogenation and transamination, has been suggested by Linderstrøm-Lang (17). More recently Herbst and Shemin (18) have actually demonstrated the *in vitro* synthesis of *dl*-alanylalanine from pyruvylalanine by non-enzymatic transamination in aqueous solution and have presented an attractive scheme for biological peptide synthesis from keto and amino acids. This scheme complements the classical concept of peptide synthesis and is consistent with the findings of Schoenheimer and his coworkers on the lability of the peptide link. The schemes of Linderstrøm-Lang and of Herbst and Shemin possess features which are consistent with the facts presented in this paper, in addition to providing a *direct* rôle for transamination in protein synthesis. Thus the fact that transaminase activity increases *ahead* of protein synthesis in the germinating oat seedling is in keeping with the above. Since an active carbohydrate metabolism makes its appearance in the germinating oat seedling at about the same time that the rate of protein synthesis increases rapidly (13), a ready source for α-keto acids and possibly glyoxals is provided. While it is not certain that glyoxals are normal metabolic intermediates, it has been shown that germinating peas and beans are capable of converting hexose diphosphate to methylglyoxal (19), and that this activity increases with time of germination. The apparent metabolic relationship of protein synthesis, carbohydrate metabolism, and transamination in the germinating oat seedling is thus in keeping with the above schemes. In assessing the possible rôle of transamination in intermediary metabolism the schemes of Linderstrøm-Lang and of Herbst and Shemin offer a more reasonable working hypothesis than the suggestion that transamination merely serves to rearrange or interconvert amino acids.

SUMMARY

1. The transamination reaction

(1) *l*(+)-Glutamic acid + oxalacetic acid $\underset{b}{\overset{a}{\rightleftharpoons}}$ α-ketoglutaric acid + *l*(−)-aspartic acid

has been studied in homogenates of developing oat embryos. The optimum pH of the system is 8.6. Reaction 1, *a* proceeds at a rate 3 times as fast as Reaction 1, *b*.

2. Transaminase activity and non-protein nitrogen (75 per cent of which is amino nitrogen) increase more rapidly than total protein during the first 72 hours. After this time they increase at parallel rates up to 120 hours.

3. $Q_{\text{transamination}}$ values of oat embryos, calculated on the basis of protein content, increase with time and reach values of the order of 900 after 96 hours development.

4. The relationship of transamination to protein synthesis is discussed.

BIBLIOGRAPHY

1. Braunstein, A. E., and Kritzmann, M. G., *Enzymologia*, **2**, 129 (1937).
2. Virtanen, A. I., and Laine, T., *Nature*, **141**, 748 (1938).
3. Cedrangolo, F., and Carandante, G., *Arch. sc. biol.*, **26**, 369 (1940).
4. Cohen, P. P., *Federation Proc.*, **1**, 273 (1942).
5. Kritzmann, M. G., *Nature*, **143**, 603 (1938).
6. Cohen, P. P., *Biochem. J.*, **33**, 1478 (1939).
7. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
8. Cohen, P. P., *J. Biol. Chem.*, **136**, 585 (1940).
9. Cohen, P. P., and Hekhuis, G. L., *J. Biol. Chem.*, **140**, 711 (1941).
10. Kaiser, S., and Albaum, H. G , *Am. J. Bot* , **26**, 749 (1939).
11. Krebs, H. A., *Biochem. J* , **32**, 108 (1938).
12. Albaum, H. G., Donnelly, J , and Korkes, S., *Am. J. Bot* , **29**, 388 (1942).
13. Albaum, H. G., and Eichel, B., *Am J. Bot.*, **30**, 18 (1943).
14. Cohen, P. P., and Hekhuis, G. L., *Cancer Research*, **1**, 620 (1941).
15. Chibnall, A. C., Protein metabolism in the plant, New Haven (1939).
16. Vickery, H. B., Pucher, G. W , Schoenheimer, R , and Rittenberg, D., *J. Biol. Chem.*, **135**, 531 (1940).
17. Linderstrøm-Lang, K., Annual review of biochemistry, Stanford University, **8**, 37 (1939).
18. Herbst, R. M., and Shemin, D , *J. Biol. Chem.*, **147**, 541 (1943).
19. Neuberg, C., and Kobel, M , *Biochem. Z.*, **229**, 433 (1930).

ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF CEREBRAL TISSUE

By WILLIAM E. STONE

(*From the Department of Surgery, Wayne University College of Medicine, Detroit*)

(Received for publication, March 25, 1943)

Analysis of cerebral tissue after fixation *in situ* with liquid air (7) seems to offer the nearest presently available approach to the chemical composition of the brain in the living animal. Studies of acid-soluble phosphorus compounds by this method have demonstrated the presence of inorganic phosphate, phosphocreatine, adenosine triphosphate, and possibly guanosine triphosphate (7, 9, 18). There still remains a quantity of organic acid-soluble phosphorus which has not been chemically identified. In a previous paper (18) this was tentatively designated "hexose phosphate."

The present paper describes an improved method for the separation and determination of the acid-soluble phosphorus compounds. The unidentified fraction has been found to include at least three substances, (*a*) one which behaves like hexose-6-monophosphate, (*b*) one which is thought to be aminoethyl phosphate, and (*c*) one which is ethanol-soluble and probably corresponds to a substance isolated from cerebral tissue by Booth (1). The revised method includes a procedure for the quantitative determination of ribose of the nucleotides or their decomposition products.

Analytical Methods

Determination of Phosphorus—The method of Fiske and Subbarow (5) has been adapted for use with the photoelectric colorimeter (Coleman universal spectrophotometer).

Readings are taken at a wave-length of 750 mμ, against a reagent blank. Under standardized conditions of temperature and time the optical density is strictly proportional to the phosphate concentration over the full range of the instrument. It has been found satisfactory to allow the color development to take place at room temperature, the readings being taken after a measured time interval: 33 ± 3 minutes at 20°, 20 ± 3 minutes at 25°, 12 ± 3 minutes at 30°, or interpolated values. Either sulfuric or hydrochloric acid may be used.

For the determination of total phosphorus in the different fractions, aliquots are digested with sulfuric and nitric acids (5, 18). Usually 1 cc. of 10 N sulfuric acid is used, with dilution to a final volume of 20 cc. for the color development. This is suitable for amounts of phosphorus up to 0.08 mg.

For all other phosphate determinations a final volume of 10 cc. is used, covering a range of 0.002 to 0.04 mg. of phosphorus.

Ribose Component of Nucleotides—Bial's reagent[1] can be used for the quantitative microdetermination of the pentose content of a trichloroacetic acid filtrate. Such tissue extracts may contain several nucleotides, all of which upon heating in strong acid solution are hydrolyzed with the formation of ribose monophosphate, and which would therefore all give the same color intensity per mole when treated with Bial's reagent. In addition, there may be small amounts of nucleoside or of free pentose in some specimens, but the pentose-containing substances present in normal brain tissue frozen *in situ* are precipitated as calcium salts by alkaline 80 per cent ethanol, and therefore are thought to be nucleotides.

A standard solution of adenosine triphosphate was prepared as follows: A trichloroacetic acid filtrate of fresh rabbit skeletal muscle was neutralized with calcium hydroxide, and ethanol added to 10 per cent. The precipitate, containing adenosine triphosphate, was dissolved in a little hydrochloric acid and the nucleotide precipitated with uranyl acetate as described by Kerr (8). The precipitate was dissolved in a few drops of 1 N hydrochloric acid, diluted to a suitable volume, and standardized by determination of the purine nitrogen (8). The solution contained 0.0173 mg. of purine nitrogen per cc., which corresponds to 0.0230 mg. of adenosine triphosphate phosphorus or 0.0077 mg. of ribose monophosphate phosphorus per cc. Determination of the acid-hydrolyzable phosphorus (10 minutes at 100° in 1 N HCl) gave 0.0152 mg. per cc., in close agreement with the theoretical two-thirds of the adenosine triphosphate phosphorus. The solution contained inorganic phosphate from the muscle and a trace of "residual organic phosphorus" (probably hexose monophosphate).

Suitable dilutions of this standard were prepared. To 3 cc. aliquots were added 6 cc. portions of Bial's reagent. The tubes were heated in a boiling water bath, cooled, and read in the photoelectric colorimeter. It was found that full color intensity was reached after a 10 minute heating period, with no further change on heating for another 10 minutes. Therefore a heating period of 10 minutes was adopted as standard procedure. The transmittance curve showed a minimum at 680 mμ. This wave-length was chosen for the readings, which were taken against a reagent blank. The optical density was found to be proportional to the concentration of the nucleotide over the full range of the instrument.

For the determination, a suitable aliquot of the unknown solution is diluted to 3 cc. and heated with 6 cc. of Bial's reagent as above. All results have been calculated as ribose monophosphate phosphorus, by use

[1] Prepared by dissolving 200 mg. of orcinol in 56 cc. of concentrated hydrochloric acid and adding 0.2 cc. of 10 per cent ferric chloride.

of the proportionality constant derived from the determinations on standard solutions. The procedure is satisfactory over a range of 0.0004 to 0.004 mg. of ribose monophosphate phosphorus. The constant must be determined for the instrument used. The value found in this laboratory has been checked with a second standard solution prepared from rabbit muscle.

Bial's reagent darkens on standing, but tests with standard solutions showed that the intensity of color development at 680 mμ, as determined against a reagent blank, remains constant over a 10 day period.

Tests on a solution of *d*-arabinose indicated that this pentose is not a suitable standard for the determination. The transmittance curve obtained was indistinguishable from that of the adenosine triphosphate standard, but the rate of color development was much slower, giving only 68 per cent as high an optical density per mole after a 10 minute heating period, and 77 per cent after 20 minutes.

EXPERIMENTAL

Conditions for Separation of Nucleotides—In attempts to adapt the previously described fractionation method (18) for use with the photoelectric colorimeter and to carry out further separations, it was found that the precipitation of adenosine triphosphate by calcium hydroxide was unsatisfactory. Further study was therefore made of the conditions required for this precipitation.

Trichloroacetic acid extracts were prepared from dog cerebral tissue frozen *in situ*. To each aliquot was added a weighed amount of finely ground calcium hydroxide not quite sufficient to neutralize the acid present, and the neutralization was completed by the dropwise addition of a 1 per cent suspension of calcium hydroxide to a pale pink phenolphthalein endpoint. Different amounts of excess 1 per cent calcium hydroxide were then added as indicated in Table I. In order further to decrease the solubility of the precipitate, neutralized ethanol was added to a concentration of 10 per cent in every case, previous experiments having shown that this does not cause precipitation of any phosphocreatine. Various temperatures and time periods were allowed for the precipitation, as indicated in Table I. After centrifuging and washing, the precipitates were dissolved in dilute hydrochloric acid and the fractions analyzed.

The results are presented in Table I. The addition of excess calcium hydroxide decreased the amount of acid-hydrolyzable phosphorus in the solution, while more inorganic, acid-hydrolyzable, and ribose monophosphate phosphorus appeared in the precipitate. It is evident that in the presence of excess calcium hydroxide the adenosine triphosphate is slowly hydrolyzed, forming inorganic phosphate and adenylic acid. This reaction has previously been observed by Lohmann (11). The adenylic acid formed

TABLE I

Precipitation of Cerebral Nucleotides by Calcium Hydroxide

Dog No.	Excess 1 per cent $Ca(OH)_2$ per 10 cc. filtrate	Conditions of pptn.	Ppt.			Solution	
			Inorganic P	Nucleotide P		Ribose monophosphate P	Acid-hydrolyzable P
				Ribose monophosphate	Acid-hydrolyzable		
	cc.		*mg. per 100 gm.*	*mg per 100 gm.*	*mg per 100 gm*	*mg. per 100 gm.*	*mg. per 100 gm.*
12	0	1 hr. at 25°	8.0	5.0	9.7	3.9	7.3
	0.1	1 " " 25°	9.1	6.2	12.8	2.6	2.4
	0.2	1 " " 25°	10.1	6.3	12.6	2.9	2.0
	0.3	1 " " 25°	10.4	5.4	11.2	3.6	2.8
	1.0	1 " " 25°	11.8	6.4	12.2	3.4	1.2
13	0	¼ " " 0°	8.8	4.2	8.4	6.4	9.8
	0	18 hrs. at 10°	8.9	6.1	12.1	4.5	6.0
	0.15	¼ hr. at 25°	10.9	6.4	12.3	4.1	4.1
	0.15	1½ hrs. at 10°	10.1	7.0	14.4	3.5	2.1

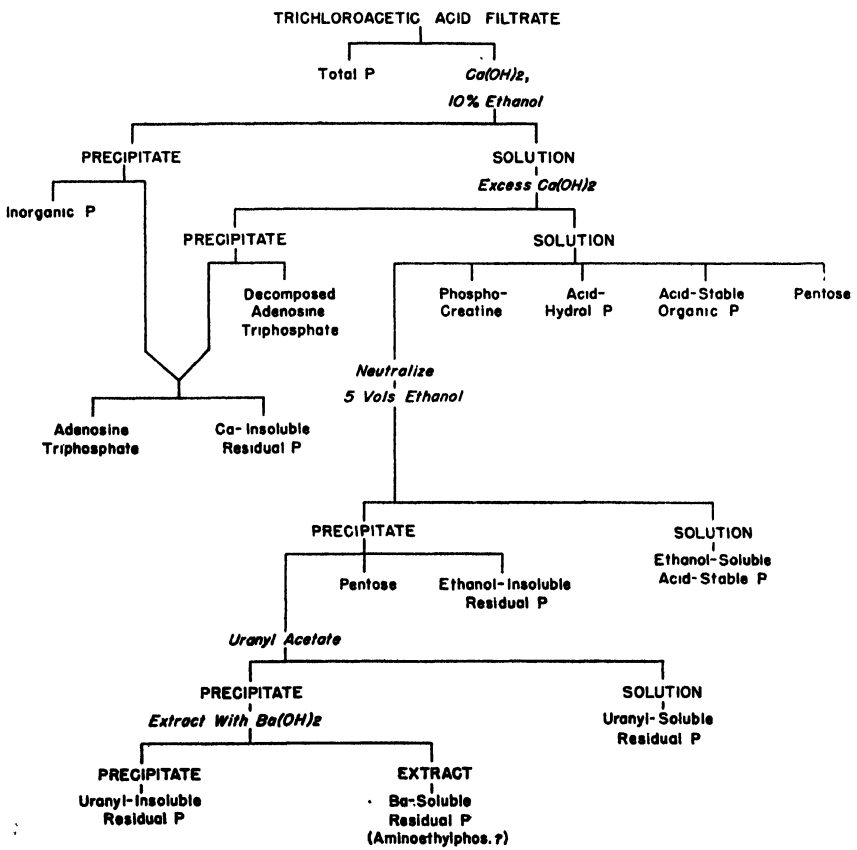


FIG. 1. Outline of fractionation of acid-soluble phosphorus compounds

passes into the centrifugate. On the other hand, if the solution is not sufficiently alkaline, the precipitation of undecomposed adenosine triphosphate is not quantitative and appreciable quantities of acid-hydrolyzable phosphorus appear in the centrifugate, as Kerr has recently pointed out (9). These conclusions have been confirmed by other experiments in which the precipitated calcium adenosine triphosphate was dissolved and reprecipitated in the presence of varying amounts of excess calcium hydroxide. A previously reported experiment of this type (18) showing complete precipitation could not be confirmed.

The substitution of barium hydroxide for calcium hydroxide gave somewhat variable results, but here also part of the nucleotide remained in the solution. The use of barium hydroxide was not further investigated.

The procedure finally adopted for the separation, outlined in Fig. 1, reduces the solubility of the adenosine triphosphate to a minimum and provides a means of correcting for the small amount of decomposition which occurs.

Fractionation Method

Inorganic Phosphate and Adenosine Triphosphate—An approximately 1:10 extract of the tissue is prepared with iced 5 per cent trichloroacetic acid as previously described (18). To a 20 cc. aliquot of the filtered solution in a short tapered centrifuge tube are added 2 drops of 0.1 per cent phenolphthalein and a previously weighed quantity of finely ground calcium hydroxide (about 200 mg.) almost sufficient to neutralize the acid present. The neutralization is completed to a pale pink by the dropwise addition of a 1 per cent suspension of calcium hydroxide. Neutral ethanol is added to 10 per cent, the end-point readjusted if necessary, and the tube allowed to stand in ice water for 15 minutes. It is then centrifuged and the solution decanted into another centrifuge tube. The precipitate is washed with 2 cc. of a calcium trichloroacetate-10 per cent ethanol solution prepared by carrying out a blank precipitation, and the washings added to the centrifugate.

The precipitate so obtained contains the inorganic phosphate and a large proportion of the adenosine triphosphate. It is dissolved in a few drops of 1 N hydrochloric acid and diluted to 10 cc. Inorganic phosphate is determined on a 1 cc. aliquot. A slight turbidity may occur in this solution but may be removed by centrifuging during the period of color development. The remainder of the solution is reserved for the determination of nucleotides.

To the centrifugate from the first precipitation, 0.1 volume of the 1 per cent calcium hydroxide suspension is immediately added. The tube is allowed to stand in ice water for 1 hour, centrifuged, decanted, and the precipitate washed with 2 cc. of the wash solution to which has been

added 0.1 volume of 1 per cent calcium hydroxide. This precipitate contains the remainder of the undecomposed adenosine triphosphate, together with a small amount of inorganic phosphate which has been formed by the decomposing action of the calcium hydroxide. The precipitate is dissolved in a few drops of 1 N hydrochloric acid and diluted to 10 cc. Determination of the inorganic phosphate on a 2 cc. aliquot permits calculation of the amount of adenylic acid formed and carried over into the soluble fraction, since two of the three phosphate groups have been split off.

Equal volumes of the two redissolved precipitates are mixed, and the following aliquots taken, 1 cc. for total phosphorus, 1 cc. for acid-hydrolyzable phosphorus, and 0.4 cc. for ribose determinations. Acid-hydrolyzable phosphorus is calculated as the difference between the amount found in the combined precipitates after acid hydrolysis (10 minutes at 100° in 1 N HCl) and the inorganic phosphate in the first precipitate, and needs no correction. To the ribose monophosphate phosphorus is added half the inorganic phosphate present in the second precipitate, which value corresponds to the amount of adenylic acid lost to the centrifugate through decomposition of adenosine triphosphate.

The "residual organic phosphorus" in the combined calcium precipitates is calculated by subtracting the inorganic, acid-hydrolyzable, and uncorrected ribose monophosphate phosphorus from the total phosphorus in this fraction.

Analysis of Soluble Fraction—The solution remaining after separation of the insoluble calcium salts is diluted to 32 cc. and the following aliquots taken, 2 cc. for phosphocreatine (6), 1 cc. for pentose, 2 cc. for acid-hydrolyzable phosphorus, and 2 cc. for total phosphorus determinations.

Fractionation with 80 Per Cent Ethanol—A 24 cc. aliquot of the soluble fraction is neutralized to a faint pink with trichloroacetic acid. 5 volumes of cold 95 per cent ethanol (made faintly alkaline after addition of 2 drops of 0.1 per cent phenolphthalein per 100 cc.) are added and the end-point readjusted if necessary to a pale pink. The mixture is allowed to stand in the refrigerator overnight, and the end-point readjusted an hour before separation. The precipitate is all collected in a single 40 cc. short tapered centrifuge tube by strong centrifugation of successive portions, and washed with neutral 80 per cent ethanol.

The precipitate contains part of the "residual organic phosphorus," together with all but traces of the phosphocreatine and pentose. It is dissolved in 10 cc. of 0.1 N hydrochloric acid and diluted to 15 cc., and 1 cc. aliquots are taken for the determination of total, acid-hydrolyzable (including phosphocreatine), and ribose monophosphate phosphorus. The "residual organic phosphorus" in this and subsequent fractions is cal-

culated by subtracting the acid-hydrolyzable and ribose monophosphate portions from the total phosphorus present.

The ethanol-soluble fraction is evaporated to dryness in an air current at room temperature, dissolved in water, diluted to 15 cc., and 2 cc. aliquots are taken for determination of total and acid-hydrolyzable phosphorus. The amounts of acid-hydrolyzable phosphorus and usually of pentose in this fraction are negligible if the proper pH has been maintained during the precipitation. Any pentose in this fraction is probably free or in the form of nucleoside; under abnormal conditions such decomposition products of adenosine triphosphate might be expected to appear (9), and such is the case in brain tissue post mortem. No correction for ribose monophosphate is made in calculating the "residual organic phosphorus" of this fraction.

It is important that the precipitation with ethanol be carried out at the proper pH. If the ethanol is added without previous neutralization of the excess calcium hydroxide, the ethanol-soluble fraction is precipitated, and upon being redissolved in trichloroacetic acid is found to have lost its characteristic solubility in ethanol.

Further Fractionation with Uranyl Acetate—The ethanol-insoluble fraction may be subdivided by precipitation with uranyl acetate (8) and extraction of the precipitate with barium hydroxide (2).[2] A 10 cc. aliquot is neutralized with 2.5 N sodium hydroxide and made just acid to phenolphthalein by the dropwise addition of 2 per cent acetic acid. A 0.5 cc. portion of a saturated solution of uranyl acetate is added and at least 10 minutes allowed for precipitation. The precipitate is separated by centrifugation and washed with 2 cc. of a 1:50 dilution of the uranyl acetate solution.

The uranyl precipitate is suspended in 3 cc. of water, warmed to 50°, and 0.5 cc. of saturated barium hydroxide added. The tube is covered with Parafilm, allowed to cool to room temperature, and agitated for 1 hour. It is then centrifuged and the precipitate again extracted in the same manner, with 1 cc. of water and 0.1 cc. of barium hydroxide. After separation the precipitate is washed twice with 3 cc. portions of water.

The precipitate is dissolved in 2 drops of 1 N hydrochloric acid and diluted to 10 cc. In the phosphate determinations on this solution a trace of barium sulfate forms, but is removed by centrifugation and apparently does not cause appreciable interference.

The extract is treated with 0.25 cc. of 10 per cent sodium carbonate to precipitate the barium, diluted to 15 cc., filtered, and analyzed. Any phosphocreatine remaining undecomposed appears in the extract, while most of the nucleotide remains in the uranyl precipitate.

[2] Colowick, S. P., personal communication.

There is usually a slight decomposition of organic phosphorus with formation of inorganic phosphate during the procedures involved in fractionation with uranyl acetate and barium hydroxide.

The results of duplicate analyses are presented in Table II.

TABLE II

Fractional Analysis of Acid-Soluble Phosphorus Compounds (Mg. per 100 Gm.)

	Dog No.	Fraction pptd by calcium hydroxide					Fraction not pptd by calcium hydroxide								
			Nucleotide P								Residual P pptd by 80 per cent ethanol				
		Inorganic P	Acid-hydrolyzable	Ribose monophosphate*	Residual organic P	Reducing power†	Phosphocreatine P	Acid-hydrolyzable P	Pentose as ribose monophosphate P*	Acid-stable ethanol-soluble P	Total	Uranyl-soluble	$Ba(OH)_2$ extract	Uranyl ppt.	Reducing power of uranyl ppt †
Brain frozen *in situ*, nembutal	15	6.8	17.7	8.5	3.6		10.9	1.7	0.6	5.1	8.1	1.0	5 5	1.3	
		6.8	16.4	8.7	5 5		11.1	0.8	0.6	5.6	7.9	1.0	5 4	1.0	
	16	8.5	18.5	9.4	3 5		9.4	0.6	0 7	7.4	5.7	0.5	3.2	0.6	
		8.5	18.5	9.4	3.9		9.4	0.4	0.6	8.6	6.9	1.1	3.0	0.5	
	17	7.2	18.6	9.7	4.0		10.9	1.2	0.5	6.8	5.0	0	2.8	1.4	
		7.1	18.5	9.6	4.8		11.3	1.0	0.5	6.8	6.1	0.6	3.6	0.5	
Brain frozen *in situ*, morphine	19	8.1	16.5	8.4	5.1	3.7	10.2	0.1	0.5	3.7	6.9	1.9	4 1	0.4	1 3
		8.3	16.1	8.3	3.4	3.7	10.1	0.2	0.6	4.1	7.4	1.0	4.3	0	1.3
	21	7.2	19.7	9.8	3.7	3.7	10.8	0.9	0.8	4.7	8.4	2.0	3 8	0	1.4
		7.2	19.0	9.8	4.2	4.0	11.1	0.7	0.9	5.2	7.8	1.9	4.0	0	1.6
Brain, post-mortem	18	34.2	4.4	5.7	2.8	6.5	0.4	0 1	3.2	4.1	7.2	3.2	1 4	0	0.7
		34.9	3.9	5.6	2 6	6.5	0 5	0	3.1	5.0	7.4	3.4	1.8	0	0.6
	20	39.5	3.5	5.3	4.5	6.4	0.3	0.6	4 2	3.4	11.9	4.7	5.0	0 8	1.2
		40.1	3.5	5.6	3.9	6.8	0.4	0.3	4.2	4.7	10.8	3.6	5.2	0.9	1.4
	Rabbit No.														
Muscle	3	29.5	37.6	18.7	7.9	5.2	52.0	2.6	1.4	2.4	6.1	0.8	0.3	3.3	4 4
		29.7	36.5	18.7	8.2	5.2	54.2	0	1.5	2.1	7.3	0.4	0.1	4.8	4.3

* Corrected.

† As hexose monophosphate P.

DISCUSSION

Hexose-6-monophosphate—The residual organic phosphorus which appears in the fraction precipitated by excess calcium hydroxide and 10 per cent ethanol and the small trace usually present in the extracted uranyl precipitate may consist largely of hexose-6-monophosphate. This statement is based on determinations of the reducing power and on comparison

with the analysis of skeletal muscle[3] (Table II), the residual phosphorus of which is known to consist largely of hexose-6-monophosphate (3, 4). The amounts of residual phosphorus present agree approximately with the reducing power determined and calculated as described by Cori and Cori (3), except where postmortem autolysis has increased the reducing power of the Ca-insoluble fraction. The reducing substance in the Ca-insoluble fraction is precipitated by uranyl acetate. Determinations of reducing power of the fraction precipitated by 80 per cent ethanol gave variable results which were usually higher than would correspond to the residual organic phosphorus present.

Aminoethyl Phosphate—The "residual organic phosphorus" extracted from the uranyl precipitate by barium hydroxide is thought to consist largely of aminoethyl phosphate, since the procedure for its separation is an adaptation of that used by Colowick and Cori (2) in isolating aminoethyl phosphate from rabbit small intestine.[2] Outhouse (16) has found this substance in tumor tissue. No significant amount was found in the rabbit muscle analyzed (Table II).

Ethanol-Soluble Fraction—The amount of acid-stable organic phosphorus remaining in the 80 per cent ethanol solution varied from 4 to 8.6 mg. per 100 gm. of cerebral tissue in the experiments shown in Table II, and in one brain (Experiment 12) has been found as high as 11 mg. per 100 gm. That this represents a distinct fraction is indicated by the following experiments.

An aliquot was evaporated to dryness in an air current, redissolved in trichloroacetic acid, and the fractionation procedure repeated. Most of the phosphorus again appeared in the ethanol solution. Only 7.5 per cent appeared as acid-stable phosphorus in the ethanol precipitate, while 10 per cent decomposed to inorganic phosphate.

The fraction precipitated by 80 per cent ethanol was dissolved in trichloroacetic acid and reprecipitated by calcium hydroxide and 80 per cent ethanol with careful adjustment of the end-point. Only a trace of phosphorus remained unprecipitated, indicating that the precipitation of the insoluble fraction is quantitative.

The ethanol-soluble fraction in aqueous solution was found to remain unprecipitated in the presence of uranyl acetate. When precipitated by excess calcium hydroxide and 80 per cent ethanol, followed by uranyl acetate fractionation, it appeared in the uranyl-soluble fraction which normally contains very little acid-stable phosphorus. When the uranyl

[3] Rabbit muscle (hind leg), stimulated at the rate of approximately three times per second for 30 seconds before the blood supply was interrupted; then removed, quickly weighed, and ground with sand and iced trichloroacetic acid. Modifications of the usual dilutions and aliquots were introduced when necessary.

fractionation was repeated in the presence of added inorganic phosphate, most of the organic phosphorus was again found in the uranyl solution, but small amounts were carried into the other fractions.

The existence of an ethanol-soluble fraction has been noted in brain and other tissues by Outhouse (16), and the substance was isolated by Booth (1), who tentatively identified it as the choline ester of sphingosinephosphoric acid. Booth found that the substance was precipitated by acetone and by mercuric chloride in ethanol.

In agreement with Booth's report, it was found that addition of 5 volumes of acetone to the aqueous solution of the ethanol fraction caused precipitation of 67 per cent of the organic phosphorus. The precipitate contained nitrogen. However, no phosphorus was precipitated when the substance dissolved in absolute ethanol was mixed with 0.5 volume of a saturated solution of mercuric chloride in absolute ethanol. The substance could not be extracted from aqueous solution by petroleum ether. No inorganic phosphate was split off in aqueous solution by excess calcium hydroxide in 6 hours at room temperature, nor was the organic phosphorus precipitated. When heated to 100° with calcium hydroxide for a few minutes, 12 per cent of the phosphorus appeared in the precipitate as inorganic phosphate and 23 per cent as organic phosphorus, while 65 per cent remained in solution.

In experiments on two rabbits it was found that skeletal muscle contains not more than 2.5 mg. of ethanol-soluble phosphorus per 100 gm.

The uranyl-soluble fraction normally contains a very small amount of "residual organic phosphorus." No suggestion as to its identity can be made at this time, nor has it been proved to be distinct from other fractions.

Phosphoglyceric Acid—The method of Rapoport (17) was adapted to the photelometric determination of 0.002 to 0.008 mg. of phosphoglyceric acid phosphorus. For these small quantities it was found advantageous to reduce the concentration of the naphthoresorcinol to 0.25 mg. per cc. of concentrated sulfuric acid, and the time of heating to 20 minutes. Precipitation as the lead salt was replaced by precipitation as the calcium salt.

No phosphoglyceric acid could be detected in cerebral tissue. The solutions showed a darker color than the blanks, but the color was not blue and the transmittance curve was not the same as that obtained with phosphoglyceric acid.

Tests with known solutions showed that any phosphoglyceric acid present would be partly carried down by the first precipitation with calcium hydroxide and 10 per cent ethanol, and almost completely precipitated by the subsequent addition of excess calcium hydroxide.

Triose Phosphate and Phosphopyruvate—An adaptation of the method

of Meyerhof and Lohmann (15) failed to detect triose phosphate in the fraction precipitated by 80 per cent ethanol. Phosphopyruvate could not be detected in this fraction by the iodoform reaction (12, 14). The low values found for acid-hydrolyzable phosphorus in the Ca-soluble fraction are additional evidence that these or other acid-labile compounds are not present in appreciable quantities.

Nucleotides—It was found that in the fraction precipitated by calcium hydroxide the corrected value for ribose monophosphate phosphorus normally equals half the acid-hydrolyzable phosphorus,[4] within the limit of error of the methods (Table II). This is to be expected from the equilibrium constants determined by Lehmann (10), according to which no measurable amounts of adenosine diphosphate (or of adenylic acid) should occur in the presence of phosphocreatine.

The amount of acid-hydrolyzable phosphorus (after subtraction of the phosphocreatine) found in the Ca-soluble fraction in normal dog brain is very small, usually less than 1 mg. per 100 gm., and may include a trace of adenosine triphosphate which has escaped precipitation. The average value found for pentose (calculated as ribose monophosphate phosphorus) in this fraction, corrected by subtracting the calculated amount resulting from action of calcium hydroxide on adenosine triphosphate, is 0.6 mg. per 100 gm., of which not more than 0.4 (half the acid-hydrolyzable phosphorus) may represent unprecipitated adenosine triphosphate. A trace of red-brown color from interfering substances is also formed in the pentose determinations on this fraction. It is clear that the amount of pentose normally occurring unassociated with acid-hydrolyzable phosphorus is extremely small, and any increase which may be observed to occur under abnormal conditions is attributable to breakdown of adenosine triphosphate.

Postmortem Changes—Two animals were killed by exsanguination, and cerebral tissue obtained for analysis about 30 minutes later. In the analyses, appropriate alterations were made when necessary in the aliquots used. The results indicate a great increase in inorganic phosphate, largely at the expense of phosphocreatine and adenosine triphosphate. In the case of Dog 20 the increase was slightly larger than can be thus accounted for; similar observations have previously been reported (13, 18). Under these conditions the hydrolysis of phosphocreatine was practically complete. The adenosine triphosphate was largely decomposed; the part precipitated by calcium hydroxide apparently consisted of adenosine diphosphate, and pentose-containing decomposition products appeared in the

[4] Kerr (9) has shown that the acid-hydrolyzable phosphorus associated with nucleotide represents mainly adenosine triphosphate, but may include a small amount of guanosine triphosphate.

Ca-soluble fraction. Approximately one-third of these was found to be soluble in 80 per cent ethanol, and probably represented nucleoside or free pentose. The part precipitated by ethanol was undoubtedly adenylic acid, since Kerr (9) has shown that no inosinic acid is formed. The present findings supplement those of Kerr on the autolytic changes in cerebral tissue.

The corrected values for the ribose monophosphate fractions have been calculated in the usual way; the results would differ by about 0.7 mg. if decomposition of adenosine diphosphate instead of the triphosphate were assumed. The amount of acid-hydrolyzable phosphorus in the Ca-insoluble fraction is slightly low even for adenosine diphosphate, but the limit of error is high where so great a value for inorganic phosphate must be subtracted. Occlusion of a small amount of adenylic acid may have occurred. The reducing power of the precipitate is unaccountably high.

A postmortem increase in the uranyl-soluble phosphorus was noted. This may have been at the expense of the alcohol-soluble fraction, but the data are not conclusive on this point.

SUMMARY

1. An improved method is presented for the fractionation of the acid-soluble phosphorus compounds of cerebral tissue.

2. A procedure is described for the determination of the ribose component of the nucleotides.

3. The unidentified organic acid-soluble phosphorus of cerebral tissue includes at least three distinct substances, (*a*) one which behaves like hexose-6-monophosphate, (*b*) one which is thought to be aminoethyl phosphate, and (*c*) an ethanol-soluble substance.

4. An analysis of the acid-soluble phosphorus compounds of skeletal muscle has been performed for comparison with the results on cerebral tissue.

5. Attempts to detect the presence of phosphoglycerate, triose phosphate, and phosphopyruvate in cerebral tissue were unsuccessful.

6. The principal changes found to occur during 30 minutes of postmortem autolysis of cerebral tissue are the hydrolysis of phosphocreatine and the partial decomposition of adenosine triphosphate. The methods used indicate the presence of adenosine diphosphate, adenylic acid, a nucleoside or free pentose, and inorganic phosphate among the decomposition products of adenosine triphosphate.

The writer is indebted to Lieutenant-Commander Charles G. Johnston, Medical Corps, United States Naval Reserve, for his encouragement in this research, to Major John E. Webster, Medical Corps, Army of the United

States, for assistance with surgical procedures, to Dr. S. Rapoport for a sample of barium phosphoglycerate, and to the Wall Chemicals Division of the Liquid Carbonic Corporation for a generous supply of liquid air.

BIBLIOGRAPHY

1. Booth, F. J., *Biochem. J.*, **29**, 2071 (1935).
2. Colowick, S. P., and Cori, C. F., *Proc. Soc. Exp. Biol. and Med.*, **40**, 586 (1939).
3. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **94**, 561 (1931–32).
4. Davenport, H. A., and Sacks, J., *J. Biol. Chem.*, **81**, 469 (1929).
5. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
6. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
7. Kerr, S. E., *J. Biol. Chem.*, **110**, 625 (1935).
8. Kerr, S. E., *J. Biol. Chem.*, **132**, 147 (1940).
9. Kerr, S. E., *J. Biol. Chem.*, **145**, 647 (1942).
10. Lehmann, H., *Biochem. Z.*, **286**, 336 (1936).
11. Lohmann, K., *Biochem. Z.*, **233**, 460 (1931).
12. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
13. MacFarlane, M. G., and Weil-Malherbe, H., *Biochem. J.*, **35**, 1 (1941).
14. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **281**, 249 (1935).
15. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934).
16. Outhouse, E. L., *Tr. Roy. Soc. Canada*, sect. 5, **29**, 77 (1935).
17. Rapoport, S., *Biochem. Z.*, **291**, 429 (1937).
18. Stone, W. E., *J. Biol. Chem.*, **135**, 43 (1940).

A NOTE ON THE IDENTITY OF A CARBONYL COMPOUND ISOLATED FROM BEEF LIVER

By ALBERT L. LEHNINGER

(From the Department of Physiological Chemistry, Medical School, University of Wisconsin, Madison)

(Received for publication, May 3, 1943)

In 1936, Cook and Harrison (1), in an investigation of the identity of the carbonyl compounds isolated from beef liver by bisulfite extraction, obtained an unknown 2,4-dinitrophenylosazone of the approximate molecular formula $C_{20}H_{20}O_{12}N_8$, which apparently accounted for the major portion of the bisulfite-binding power of liver extracts (2). They were unable to suggest a structure for this compound from their data, but remarked that it was probably a derivative of some condensation product formed in intermediate metabolism.

In the course of some work on the intermediate metabolism of fatty acids, this compound became of interest because of its possible significance as a metabolite, and work was begun on its isolation with the aim of determining its structure.

EXPERIMENTAL

Several isolations of the osazone from beef liver were carried out, according to the directions of Cook and Harrison. No difficulty whatsoever was encountered in repeating their procedure. However, their statement, "30 g. of 2:4-dinitrophenylhydrazine dissolved in 20 ml. con. HCl," should be modified, since this amount of reagent is not soluble in 20 ml. of acid, but forms a thick paste. The osazone was purified according to their procedure and the yields of the alcohol-recrystallized product varied from 1 to 4 gm. per kilo of beef liver.

The unknown osazone, which yielded a blue color with alcoholic KOH, gave decomposition points (corrected) ranging from 173–177° after recrystallization from ethyl alcohol. Cook and Harrison reported 175°. Elementary analysis showed it to be identical with their compound. Found, C 42.43, H 3.69, N 20.00 per cent. Cook and Harrison reported C 42.32, H 3.58, N 20.12 per cent.

It should be mentioned that the "recrystallization" from 97 per cent alcohol employed by Cook and Harrison usually resulted in an amorphous material in our hands. Attempts were then made to recrystallize it from another solvent to obtain true crystals. The compound was successfully recrystallized from nitrobenzene, yielding thin, fibrous microscopic needles

of an orange-red color, frequently arranged in rosettes. The crystals were washed with nitrobenzene and benzene, and dried *in vacuo* over P_2O_5. The decomposition point was found to have risen to 240–242°, suggesting that the original compound as recrystallized from alcohol still harbored impurities. Another recrystallization from nitrobenzene established the decomposition point at 255–257°, which was not altered in five further recrystallizations. Elementary analysis now yielded the following values: C 40.29, H 3.38, N 20.63 per cent.

The data at this point strongly suggested that the compound was the 2,4-dinitrophenylosazone of glucose, decomposition point 256–257°; calculated, C 40.14, H 3.34, N 20.81 per cent (3). Cook and Harrison had stated that glucose 2,4-dinitrophenylosazone could not be found in their experiments, without further comment. The analysis of the alcohol-recrystallized compound naturally did not necessarily suggest this possibility.

An authentic sample of glucose 2,4-dinitrophenylosazone was prepared by subjecting a 3 per cent solution of glucose, containing bisulfite, to the isolation procedure of Cook and Harrison, beginning at the point of addition of the hydrazine reagent. The alcohol-recrystallized product gave a decomposition point at 177°, identical with that of the natural product. On repeated recrystallization from nitrobenzene, it assumed a constant decomposition point at 255–257°. The crystal structure was identical with that of the natural derivative; mixed melting point tests showed no depression. The analysis gave C 40.08, H 3.31, N 20.62 per cent, showing it to be probably identical with the natural product. About 90 per cent of the alcohol-recrystallized, natural product could be recovered as the pure glucose derivative. The impurities, which are believed to originate in the pyridine treatment of Cook and Harrison, were tarry in nature and resisted further purification.

Further support for the conclusion that the isolated derivative is identical with glucose 2,4-dinitrophenylosazone is as follows:

Glucose has a slight bisulfite-binding capacity (2) which was found to be greatly increased on long standing. On concentration of such a solution *in vacuo* (as in the isolation procedure) the total bisulfite bound increases as expected from the data of Clift and Cook (2). The bisulfite-binding power of glucose is also destroyed by boiling with strong alkali. All these observations coincide with those made on the liver extract.

When the acidified liver extract was freed of sulfur dioxide and extracted with ether, the unknown fraction was not removed by the ether. When the remaining water solution, which contained copper-reducing and fermentable compounds, was treated with sodium acetate and phenylhydrazine hydrochloride, the typical glucose phenylosazone crystals were ob-

tained, which after recrystallization gave a melting point of 200° (authentic sample 202°), mixed melting point 200°.

Other physical properties showed the liver compound to be identical with glucose 2,4-dinitrophenylosazone. Both compounds gave identical spectral transmittance curves in dioxane solution, showing a rather broad maximum at 445 mμ. The specific extinction coefficient at 445 mμ in dioxane was 57.2 ± 2 at a concentration of 5.00×10^{-3} gm. per liter for both compounds. On treatment with alcoholic KOH both compounds gave an intense blue color, having identical absorption curves with a broad maximum at 550 mμ.

Although the deep color of dioxane solutions prevented accurate readings, the specific rotations at 25° were found to be approximately identical, in the neighborhood of −140°.

Some experiments showed that the compound could be obtained from the liver of several laboratory species and, furthermore, that the time elapsed between death of the animal and the beginning of the isolation procedure had no great bearing on the character or quantity of the compound obtained.

No other hydrazones or osazones could be found in any quantity by Cook and Harrison's fractionation procedure from several beef or rat livers. There were traces of acid hydrazones in the 10 per cent alcohol fraction, but not enough to identify. Acetic acid 2,4-dinitrophenylhydrazide was isolated in confirmation of Cook and Harrison, although the yields were smaller.

SUMMARY

An unknown 2,4-dinitrophenylosazone ($C_{20}H_{20}O_{12}N_8$) isolated from beef liver by Cook and Harrison was further purified and found to be identical with glucose 2,4-dinitrophenylosazone.

BIBLIOGRAPHY

1. Cook, R. P., and Harrison, K., *Biochem. J.*, **30**, 1640 (1936).
2. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).
3. Glaser, E., and Zuckermann, N., *Z. physiol. Chem.*, **167**, 37 (1927).

THE DETERMINATION OF ACID-SOLUBLE PHOSPHOGLYCEROL IN LIVER

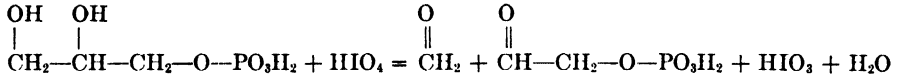
By ERNST LEVA and S. RAPOPORT

(From the Children's Hospital Research Foundation and the Departments of Pediatrics and Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, April 5, 1943)

In the course of a series of unpublished studies on the turnover of acid-soluble phosphorus in the livers of rats changes have been found to occur in a fraction which appears to be composed mainly of phosphoglycerol. The presence of α-phosphoglycerol in the liver was first demonstrated by Fiske and Subbarow (1). Further studies of the metabolism of the phosphoglycerol fraction necessitated the development of the method for its determination that is here reported, a method based on the work of Malaprade (2), Fleury and coworkers (3, 4), and Bailly (5, 6).

Fleury and Paris (3) found that periodic acid, which preferentially attacks adjacent hydroxyl groups, oxidizes α-phosphoglycerol to form formaldehyde and phosphoglycolaldehyde according to the reaction,



while β-phosphoglycerol is not attacked. In contrast to phosphoglycerol, the diose phosphate is easily hydrolyzable (4), yielding inorganic P and free diose. Thus a determination of inorganic P after a preliminary oxidation and hydrolysis may serve as a measure of α-phosphoglycerol. The β form also can be determined by means of the reaction discovered by Bailly (5, 7) that 90 per cent of the β-phosphoglycerol is converted to α-phosphoglycerol by heating in acid solution. This transformation is reversible (6), and the same equilibrium is reached from either side.

The determination of phosphoglycerol in tissues involves the following steps: After extraction of the tissue with trichloroacetic acid, interfering substances (mainly nucleotides) are removed by precipitation with mercuric acetate at pH 5 and the phosphoglycerol together with some other phosphorus compounds is then precipitated with Ba, Hg, and glycine in 70 per cent methanol at pH 9 to 10. The precipitate is decomposed and inorganic P is removed as magnesium ammonium phosphate. The α-phosphoglycerol is determined by oxidation with periodic acid and subsequent hydrolysis (heating for 1 hour in a boiling water bath with N H_2SO_4) of the phosphoglycolaldehyde to liberate inorganic P which is determined by the

method of Fiske and Subbarow (8). The sum of α- and β-phosphoglycerol is determined by heating the samples a short time in acid solution, before oxidation, to establish an equilibrium between the α- and β-phosphoglycerol. In glycogen-rich extracts only the sum of α- and β-phosphoglycerol can be determined.

Reagents—

Trichloroacetic acid, 5 and 20 per cent solutions. Keep in refrigerator.

Indicators, phenol red, 0.04 per cent, and phenolphthalein, 1 per cent, solutions.

Sodium hydroxide, 5 N and 0.2 N solutions.

Mercuric acetate reagent, 20 gm. of mercuric acetate and 2 cc. of glacial acetic acid in 100 cc. of solution.

Barium acetate, 50 gm. of $Ba(C_2H_3O_2)_2 \cdot H_2O$ in 100 cc. of solution, filtered.

Barium hydroxide solution, saturated at room temperature.

Glycine solution, 2 gm. in 100 cc. of water.

Methanol, C.P.

Sulfuric acid, 1 N, 5 N, and 10 N solutions.

Magnesium sulfate, 1 gm. of $MgSO_4 \cdot 7H_2O$ in 100 cc. of water.

Concentrated ammonium hydroxide, sp. gr. 0.90.

Sodium bisulfite, solid.

Periodate reagent, 2.94 gm. of trisodium paraperiodate, $Na_3H_2IO_6$, (G. Frederick Smith Chemical Company) in 100 cc. of 3 N H_2SO_4, giving a 0.1 M solution.

Reagents of Fiske and Subbarow (8), for the determination of P.

Procedure

1. Preparation of Trichloroacetic Acid Extracts—Remove the liver quickly from the anesthetized (nembutal) animal and drop it into dry ice-ether mixture. Remove it from the freezing mixture, weigh quickly, and then grind in a measured volume of 5 per cent trichloroacetic acid, using approximately 10 cc. of acid for 1 gm. of tissue. A Waring blendor is a valuable aid for the extraction, as it gives a homogeneous suspension in 2 minutes. Allow the mixture to stand 5 minutes and filter. In calculation of the dilution the tissue water is included.

2. Removal of Nucleotides (and Other Interfering Substances)—Measure 10 cc. of the filtrate into a graduated centrifuge tube, add 1 drop of phenol red indicator, and adjust to a faint pink, using first 5 N and then 0.2 N NaOH solution. Add 0.6 cc. of the mercuric acetate reagent, and after the contents of the tube are mixed thoroughly with a glass rod remove and rinse the rod. A flocculent precipitate appears within a minute. Place the tube in the refrigerator for 1 hour, then centrifuge, and pour off the supernatant fluid with as complete drainage as possible into a 100 cc.

centrifuge tube. The amount of fluid adhering to the precipitate, less than 0.2 cc., is disregarded.

3. Precipitation of Phosphoglycerol—To the solution in the 100 cc. centrifuge tube add 1 cc. of 50 per cent barium acetate, 0.35 cc. of 2 per cent glycine solution, and 1 drop of phenolphthalein indicator. Make the mixture alkaline with saturated barium hydroxide solution, and add 3 volumes of methanol. Mix with a glass rod, remove and rinse the rod, and place the tube in a refrigerator overnight.

4. Decomposition of Precipitate Containing Phosphoglycerol, and Removal of Inorganic P—The next day, centrifuge and discard the supernatant fluid. Disperse the precipitate in 1 cc. of 20 per cent trichloroacetic acid, add 2 cc. of N H_2SO_4, and remove the mercury by passing through the mixture a current of H_2S gas at room temperature. If the original extract is rich in glycogen, the HgS forms a colloidal suspension and the solution must be heated 20 minutes in a boiling water bath to hydrolyze the glycogen sufficiently to permit the flocculation of the HgS. Transfer the precipitate and washings to a volumetric flask or graduated centrifuge tube and bring the volume to 10 cc. After mixing, centrifuge, pour the supernatant fluid into a 50 cc. centrifuge tube, and remove the H_2S by aeration for 5 minutes. Measure 8 cc. of the fluid into a graduated 15 cc. tube, then add to it 1 cc. of 1 per cent $MgSO_4$ solution and 1 drop of phenolphthalein indicator, and adjust to a definitely alkaline reaction with concentrated ammonium hydroxide solution. Scratch the side of the vessel with a glass rod to hasten the appearance of the $MgNH_4PO_4$ precipitate, remove the rod and rinse it with a few drops of water, and bring the solution to a convenient volume, usually 11 cc. After mixing by inverting the tube, allow it to stand several hours, then centrifuge, and pipette the supernatant fluid into a dry test-tube.

5. Oxidation and Hydrolysis of Phosphoglycerol—For oxidation of the phosphoglycerol transfer an aliquot, not exceeding 4 cc., containing approximately 30 γ of phosphoglycerol phosphorus to a 10 cc. volumetric flask. Discharge the pink color with 1 or 2 drops of 10 N H_2SO_4, and then add 0.5 cc. of the periodate reagent and water to make the volume 4.5 cc. After the solution has stood 1 hour at room temperature, reduce the excess periodate by adding solid $NaHSO_3$ in small portions. At first iodine is liberated; then the solution becomes colorless as the iodine is reduced by the bisulfite. Add 0.4 cc. of 10 N H_2SO_4 and immerse the flask in a boiling water bath. After 5 minutes firmly stopper the flask with a rubber stopper and continue the heating 1 hour. Then cool the solution and determine the liberated inorganic P according to Fiske and Subbarow (8) using 1 cc. of the Molybdate III solution and 0.4 cc. of aminonaphtholsulfonic acid solution in a volume of 10 cc.

The inorganic P is multiplied by the factor 1/0.965 in order to obtain the

α-phosphoglycerol P in the aliquot. This factor was determined from the average yield of inorganic P from α-phosphoglycerol in pure solutions. In glycogen-rich extracts, which have been heated in step (4) of the procedure, an equilibrium is established between α- and β-phosphoglycerol. In this case, the value for inorganic P is multiplied by the factor 1/0.862, as described in the next paragraph, to obtain the sum of both isomers.

6. Determination of Sum of α- and β-Phosphoglycerol—Transfer an aliquot (4 cc. or less) of the fluid for analysis into a 10 cc. volumetric flask, and add 0.4 cc. of 10 N H_2SO_4 and water to make the volume 4.5 cc. Heat the solution 20 minutes in a boiling water bath, cool the flask, add 0.5 cc. of periodate reagent, and then allow the mixture to stand 1 hour at room temperature. Reduce the excess periodate and proceed with the hydrolysis and determination of inorganic P as described in step (5), except that additional H_2SO_4 need not be added for the hydrolysis. The value for inorganic P found, multiplied by the factor 1/0.862 (based on the average yield of inorganic P from pure solutions of phosphoglycerol similarly treated) represents the sum of α- and β-phosphoglycerol.

EXPERIMENTAL

Removal of Interfering Substances with Mercuric Acetate—As it was found that nucleotides interfered with the determination of phosphoglycerol, mercuric acetate in acetic acid solution was selected to remove them. This reagent was used by Kerr (9) in the preparation of adenosine triphosphate from muscle. Nucleotides are precipitated quantitatively. From liver extracts the reagent also precipitates a nitrogen-free (otherwise unidentified) phosphorus compound, while leaving all the phosphoglycerol in the supernatant fluid.

Precipitation of Phosphoglycerol—Various Pb, Cu, Hg, Ag, and Ba salts were tested for the precipitation of phosphoglycerol in the presence of different amounts of methanol, ethanol, ether, and acetone. In mixtures containing 3 parts of methanol and 1 part of ether for 1 part of aqueous solution, the precipitation of the Cu salt was 48 per cent, that of the Ba, Hg, and Ag salts 80 to 92 per cent complete. The lead salt alone was quantitatively precipitated in pure solutions of phosphoglycerol. However, only 85 per cent of phosphoglycerol added to trichloroacetic acid extracts of liver was precipitated as lead salt. At the same time it was found that precipitation with Ba and Hg together in approximately 70 per cent methanol, which permitted recovery of about 80 per cent of phosphoglycerol in pure solutions, was quantitative in the presence of liver extract. In the search for the factor, or factors, responsible for the improved recovery various nitrogenous compounds, including a number of amino acids, were tried. It was found that glycine, alanine, leucine,

cysteine, and tyrosine, the monoaminocarboxylic acids tried, were effective in improving the precipitation of phosphoglycerol. The other substances tried, namely ammonia, urea, arginine, glutamic acid, histidine, and tryptophane, did not enhance the yield. It seems probable from these data that the amino acids in the trichloroacetic acid extract of liver, perhaps chiefly glutathione, are responsible for the improved yields in its presence.

In Table I are summarized data illustrating the effect of Ba, glycine, and various concentrations of methanol on the precipitation of phosphoglycerol.

TABLE I

Effect of Ba, Hg, Glycine, Methanol, and Ether on Precipitation of Phosphoglycerol

Samples of α-phosphoglycerol, containing 0.268 mg. of P, were precipitated as described under "Procedure," with mixtures of substances indicated. The precipitates were dissolved in N HNO_3. Ba or Hg or both were removed, and the P was determined in an aliquot.

Substances added					Recovery of P in ppt
Ba	Hg	Glycine	Methanol	Ether	
mM	*mM*	*mM*	*vol*	*vol*	*per cent*
1.8	0.3	0.09	3	1	99.5
1.8	0.3	0.09	3		100.3
1.8	0.3	0.09	2	1	95.5
1.8	0.3	0.09	2		80.7
1.8	0.3	0.09	1		48.1
1.8	0.3	0.09			0
1.8	0.3		3	1	83.5
	0.3	0.09	3	1	57.5

Standards—Two preparations of α-phosphoglycerol were used interchangeably for the making of standard solutions. For one sample of barium α-phosphoglycerol we are indebted to Dr. J. J. Rae of the Department of Chemistry of the University of Toronto. Another sample was prepared in our own laboratory from sodium β-phosphoglycerol (Eastman) according to the procedure of Bailly (5). The purity of both preparations was about the same, over 99 per cent, determined by a titration procedure similar to that of Voris *et al.* (10). For standard solutions of β-phosphoglycerol the Eastman product was used without further purification, since it proved to be free of α isomer.

Time of Oxidation and Hydrolysis—Solutions of α-phosphoglycerol were incubated at room temperature with periodate for varying periods of time and the excess periodate was determined according to the method of Rappaport *et al.* (11). It was found that 40 minutes were necessary for the reaction to become complete. The time required to hydrolyze the

oxidation product, phosphoglycolaldehyde, was tested in another series of experiments, presented in Table II. Based on these data 1 hour's oxidation time followed by 1 hour's hydrolysis in N H_2SO_4 in a boiling water bath

TABLE II

Liberation of Inorganic P from α-Phosphoglycerol by Hydrolysis after Periodate Oxidation

A standard solution of α-phosphoglycerol was oxidized with periodate as described under "Procedure," the excess periodate was removed with $NaHSO_3$, and H_2SO_4 was added to make the solution 1 N. The mixture was heated in a boiling water bath for the periods indicated, before the determination of inorganic P.

Hydrolysis time	Inorganic P found
min.	*per cent total P*
4½	24.4
10	48.0
15	60.5
25	81.1
40	93.5
60	96.5*
80	96.3

* Average of eight determinations, varying within ±2 per cent.

TABLE III

Conversion of α- and β-Phosphoglycerol by Heating in Acid Solution

Solutions of α- and β-phosphoglycerol were heated in N H_2SO_4 in a boiling water bath for varying periods of time as indicated. After cooling, they were oxidized, hydrolyzed, and the inorganic P was determined as described under "Procedure."

Substance heated	Time of heating	α-Phosphoglycerol
	min.	*per cent total P**
β-Phosphoglycerol	5	67.1
	10	87.2
	15	89.4
	25	88.9
	40	89.4
α-Phosphoglycerol	20	89.3†

* This value was calculated on the assumption that pure α-phosphoglycerol yields 96.5 per cent of its P as inorganic phosphate under the conditions of the experiment.

† Average of eight determinations, varying within ±2 per cent.

was chosen as routine procedure, giving an average yield of 96.5 per cent of inorganic P from pure α-phosphoglycerol (see Table II).

Transformation of α- and β-Phosphoglycerol into Each Other—The conversion of β- into α-phosphoglycerol after varying periods of heating with

TABLE IV

Influence of Other Phosphorus Compounds on Determination of α-Phosphoglycerol

Solutions of the compounds indicated in the table were precipitated with Ba, Hg, glycine, and 3 volumes of methanol as described under "Procedure." The precipitates were dissolved, made up to 10 cc., and total P and inorganic P were determined after oxidation and hydrolysis.

Material analyzed	P in sample	α-Phosphoglycerol P found	Total P found
	mg.	*mg.*	*mg*
Monophosphoglyceric acid	0.268	0	0.277
Diphosphoglyceric acid	0.478	0.01 (?)	0.495
Same with addition of 0.268 mg. of P of α-phosphoglycerol	0.746	0.271	
Fructose diphosphate	0.595	0.386	0.607
Same hydrolyzed without previous oxidation	0.595	0.406	0.607
β-Phosphoglycerol	0.239	0.01 (?)	0.239

TABLE V

Recovery of α-Phosphoglycerol from Pure Solutions and in Presence of Trichloroacetic Acid Extracts of Liver

Pure solutions, liver extracts from both fed and fasted rats, and liver extracts to which known amounts of α-phosphoglycerol had been added were analyzed as described under "Procedure."

Material analyzed	Inorganic P found	α-Phosphoglycerol P × f_1*	Recovery of added P	Total P found	Recovery
	mg.	*mg.*	*per cent*	*mg.*	*per cent*
10 cc. trichloroacetic acid extract, corresponding to 1.02 gm. liver, rich in glycogen	0.244	0.283		0.372	
	0.248	0.287		0.376	
Same with addition of 0.209 mg. of P of α-phosphoglycerol	0.428	0.496	101.4	0.582	99.7
	0.431	0.500	103.2	0.587	102.1
Standard solution of α-phosphoglycerol containing 0.209 mg. P	0.177	0.205	98.3	0.204	97.8
	0.177	0.205	98.3	0.203	97.4
		× f_2†			
10 cc. trichloroacetic acid extract, corresponding to 1.23 gm. liver, free of glycogen	0.185	0.192		0.288	
Same with addition of 0.209 mg. of P of α-phosphoglycerol	0.437	0.453	97.4	0.557	100.4
	0.448	0.464	101.5	0.548	97.0
Standard solution of α-phosphoglycerol containing 0.209 mg. P	0.193	0.200	96.0	0.206	98.8
	0.196	0.203	97.4	0.204	97.8

* The values for inorganic P were multiplied by 1/0.862 to correct for the average yield of inorganic P from pure α-phosphoglycerol after preliminary heating. This procedure is necessary in extracts containing large amounts of glycogen.

† The values for inorganic P were multiplied by the factor of 1/0.965 to correct for the average yield of inorganic P from pure α-phosphoglycerol solutions on oxidation and hydrolysis.

N H_2SO_4 in a boiling water bath is shown in Table III. Equilibrium was established in less than 15 minutes when the mixture contained about 89 per cent and 11 per cent of the α and β forms, respectively. Table III also shows the effect of heating solutions of α-phosphoglycerol in N H_2SO_4 for 20 minutes preliminary to the oxidation with periodate. It is evident that the same equilibrium is reached whether one starts with pure α- or β-phosphoglycerol.

Influence of Other Acid-Soluble P Compounds—Mono- and diphosphoglyceric acid, fructose diphosphate, and β-phosphoglycerol were tested for possible interference with the method. The results are shown in Table IV.

TABLE VI

Acid-Soluble Phosphoglycerol Content of Various Tissues

Tissue samples were collected and analyzed as described under "Procedure."

Tissue analyzed	α-Phosphoglycerol	Sum of α- and β-phosphoglycerol	β-Phosphoglycerol
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
Rat (fed) liver*		27.8	
" (fasted) liver†	14.8		1.9
" kidney‡	7.9		0.3
" brain‡	5.8		0
" heart‡	11.0		1.6
" intestine	8.1		0
Rabbit (fed) liver		28.9	
" kidney	6.7		1.1
" heart	8.3		1.8
" muscle	4.1		
Pigeon (fed) liver		27.8	

* Average of sixteen determinations.

† Average of eight determinations.

‡ Pooled samples from three animals.

It was found that the glyceric acid esters and β-phosphoglycerol did not interfere. The amounts of inorganic P released from fructose diphosphate by hydrolysis, after incubation with periodate, were found to be the same as those liberated in comparable periods of acid hydrolysis alone. Therefore, satisfactory values for phosphoglycerol determined in the presence of fructose diphosphate should be obtained if the amounts of the inorganic P found after oxidation and hydrolysis are corrected for the inorganic P found after acid hydrolysis alone.

Results

In Table V are presented data on the recovery of α-phosphoglycerol from pure solutions, carried through the entire procedure, and on its recovery

when known amounts of α-phosphoglycerol were added to both glycogen-rich and glycogen-free extracts of rat liver. The values for inorganic P found in extracts containing glycogen after oxidation and hydrolysis, and in the standard solutions similarly treated, were multiplied by the factor 1/0.862, based on the average yield of inorganic P from phosphoglycerol heated in acid solution before oxidation. The values for inorganic P in the glycogen-free extracts and standard solutions similarly treated were multiplied by the factor 1/0.965, based on the average yield of inorganic P obtained from phosphoglycerol after oxidation and hydrolysis. The recovery of phosphoglycerol was 96 to 99 per cent in pure solutions, and 97 to 103 per cent in the experiments in which various substances were added. The recoveries in terms of total phosphorus were in the same range.

In Table VI are given some representative results on the acid-soluble phosphoglycerol content of the livers of rats, rabbits, and pigeons, and of some other tissues of rats and rabbits. Liver is by far the richest tissue, the livers of fed rats and rabbits containing over 25 mg. of phosphoglycerol per 100 gm. Livers of fasted rats contained significantly lower amounts. Little or no acid-soluble β-phosphoglycerol was found in any of the tissues studied. The effects of diet on the distribution of P compounds in the liver will be reported in detail later.

SUMMARY

A method for the determination of acid-soluble α- and β-phosphoglycerol in liver and other tissues is described, and results on the phosphoglycerol content of some tissues are presented.

BIBLIOGRAPHY

1. Fiske, C. H., and Subbarow, Y., *Science*, **70**, 381 (1929).
2. Malaprade, L., *Compt. rend. Acad.*, **186**, 382 (1928).
3. Fleury, P., and Paris, R., *J. pharm. et chim.*, **18**, 470 (1933).
4. Fleury, P., and Courtois, J., *Compt. rend. Acad.*, **209**, 219 (1939).
5. Bailly, M. C., *Compt. rend. Acad.*, **206**, 1902 (1938).
6. Bailly, M. C., *Compt. rend. Acad.*, **208**, 443 (1939).
7. Verkade, P. E., Stoppelenburg, J. C., and Cohen, W. D., *Rec. trav. chim. Pays-Bas*, **59**, 886 (1940).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Kerr, S. E., *J. Biol. Chem.*, **140**, 77 (1941).
10. Voris, L., Ellis, G., and Maynard, L. A., *J. Biol. Chem.*, **133**, 491 (1940).
11. Rappaport, F., Reifer, I., and Weinmann, H., *Mikrochim. acta*, **1**, 290 (1937).

THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE LIVERS OF RATS, FED AND FASTING

By S. RAPOPORT, ERNST LEVA, AND GEORGE MARTIN GUEST

(From the Children's Hospital Research Foundation and the Departments of Pediatrics and Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, April 5, 1943)

In a previous study (1) dealing with the distribution of acid-soluble P in the liver we reported some data on differences between fed and fasted rats with regard to concentrations of the inorganic, the easily hydrolyzable, and the "rest" organic P. In the study here reported the effect of varying periods of fasting was investigated, and a further differentiation of the organic P was attempted, based on steps involved in the determination of phosphoglycerol. The acid-soluble P was divided as follows: (1) inorganic P; (2) easily hydrolyzable P, representing 67 per cent of the adenosine triphosphate plus 50 per cent of the adenosine diphosphate; (3) "mercuric-insoluble" P, containing all the stable nucleotide P, which accounted for 50 to 60 per cent of this fraction; (4) the sum of α- and β-phosphoglycerol; (5) residual P, representing substances precipitated together with phosphoglycerol; (6) alcohol-soluble P, compounds yielding Hg and Ba salts soluble in 80 per cent alcohol. This fractionation procedure gave reproducible results, the sum of the fractions usually agreeing with the total acid-soluble P within less than 5 per cent. Attempts to identify chemical compounds among the various fractions are under way.

Methods

Male rats weighing 200 $\pm$ 45 gm. from the stock of Sprague-Dawley, Inc., were used in all experiments. They were kept on the stock diet of the laboratory. Rats designated as fed had free access to food at all times, while food was withdrawn from the fasted animals at varying times stated in Tables I to III before they were killed. The animals were anesthetized by intraperitoneal injection of nembutal, and the liver was quickly removed and dropped into a mixture of dry ice and ether. The frozen organ was weighed, and a trichloroacetic acid extract was prepared as described elsewhere (2).

Fractionation of Extract—The inorganic P and the total acid-soluble P were determined in the usual manner (3). The easily hydrolyzable P was determined by heating the extracts for 15 minutes in a boiling water bath with N sulfuric acid. The other fractions were determined in a common sample as follows: 10 cc. of extract were treated with mercuric acetate as

described in the phosphoglycerol method (2). After standing for 1 hour in the refrigerator, the material was centrifuged and the supernatant fluid was transferred to a 100 cc. centrifuge tube. The precipitate was dissolved in 3 cc. of N H_2SO_4, Hg was removed with a current of H_2S gas, and the mixture was heated in a boiling water bath for 15 minutes to hydrolyze the nucleotide polyphosphates. 1 cc. of 1 per cent $MgSO_4$ was added, and the inorganic phosphate was precipitated as magnesium ammonium phosphate. After standing overnight the tubes were centrifuged, the precipitate was washed once with diluted ammonium hydroxide, the combined supernatant fluids were made to a definite volume, and the total P was determined in a convenient aliquot. The stable P of adenosine di- and triphosphate and probably adenylic acid accounts for 50 to 60 per cent of the P in this fraction, designated as "mercuric-insoluble." In addition at least one other nitrogen-free compound is present. To the supernatant fluid of the mercury precipitate, barium acetate, glycine, and methanol were added. The resulting precipitate was decomposed, the inorganic P was removed, and the phosphoglycerol P and total P of this fraction were determined (2). The difference between the total P and the phosphoglycerol P represents the residual P. The supernatant fluid of the precipitate containing phosphoglycerol was evaporated, barium and Hg were removed, and the total P in this fraction, designated as alcohol-soluble P, was determined. This fraction probably contains the compound thought to be choline sphingosine phosphate, isolated by Booth (4) from kidney, and by Strack *et al.* (5) from the liver of cattle.

These fractions together accounted for about 95 to 102 per cent of the independently determined total acid-soluble P.

The data were analyzed statistically according to the methods of Fisher (6).

Results

In the first two rows of Table I are presented the mean concentration values of the total acid-soluble P and of its fractions in the livers of fed rats and of rats fasted for 24 hours. Differences between these two groups and estimates of their significance are summarized in the first row of Table II. The data indicate that fasting produced a small, but statistically significant, decrease of the total acid-soluble P. The inorganic P was markedly increased, while the easily hydrolyzable and the phosphoglycerol fractions were greatly diminished. Of the other fractions, only the residual P seemed to show a significant change. It is of interest to compare these results with experiments reported previously (1) in which another strain of animals, maintained on a different diet, was used and a different method was employed to prepare the trichloroacetic acid extracts. The values for the fed

rats are much the same in both series. The fasted rats of the present series, on the other hand, show considerably higher values of inorganic P and lower ones of the easily hydrolyzable P. In the previous series the fasted group had been deprived of food for 16 to 18 hours, while in the experiments here reported the animals were fasted for 24 hours. The explanation suggested itself that the differences found between the two series might be accounted for by the varying periods of fasting. This would imply that important changes in the concentrations of the various fractions of phosphorus take place in the period between 16 and 24 hours of fasting. To examine

TABLE I

Mean Concentrations of Acid-Soluble P in Livers of Rats

	Total acid-soluble P, mean and S E.	Inorganic P, mean and S E	Easily hydrolyzable P, mean and S.E.	Phosphoglycerol P; mean and S E	"Mercuric-insoluble" P; mean and S E.	Residual P; mean and S E	Alcohol-soluble P; mean and S.E.	Sum of fractions; mean and S.E.
	mg. per 100 gm	*mg. per 100 gm.*	*mg. per 100 gm*	*mg. per 100 gm.*	*mg. per 100 gm*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*
Fed	103.9 ±1.7	17.8 ±0.6	15.8 ±0.5	27.8 ±0.8	27.5 ±0.4	5.8 ±0.7	6.7 ±0.5	101.4 ±1.5
No. of determinations	20	16	16	16	10	16	16	
Fasted 24 hrs.	98.2 ±0.9	29.3 ±0.6	10.4 ±0.3	16.5 ±0.5	28.6 ±0.5	3.5 ±0.6	5.8 ±0.3	94.1 ±1.2
No. of determinations	34	20	20	34	16	20	20	
Fasted 48 hrs. or more	89.2 ±1.2	26.3 ±0.9	10.7 ±0.5	18.2 ±0.5	27 9 ±0.4	1.6 ±0.3	4.3 ±0.3	89.0 ±1.3
No. of determinations	13	13	13	13	13	13	13	

this point further, a number of rats were fasted for periods of time varying from 12 to 96 hours.

In Table III are presented the individual data on the distribution of phosphorus in the livers of rats fasted for varying periods of time. They indicate that the inorganic P was increased as early as 12 hours after the withdrawal of food to reach maximum levels after 24 hours. In animals fasting 48 hours or longer it appears somewhat decreased as compared with the 24 hour level. The easily hydrolyzable P and the phosphoglycerol P, following similar patterns of change, were at their lowest levels after 24 hours, and did not change further during the more prolonged periods of

TABLE II

Significance of Differences between Mean Concentrations of Acid-Soluble P in Livers of Rats

	Total acid-soluble P		Inorganic P		Easily hydrolyzable P		Phosphoglycerol P		"Mercuric-insoluble" P		Residual P		Alcohol-soluble P	
	Difference	p*	Difference	p*	Difference	p*	Difference	p*	Difference	p*	Difference	p*	Difference	p*
	mg. per 100 gm.		*mg. per 100 gm.*		*mg. per 100 gm.*		*mg. per 100 gm.*		*mg. per 100 gm.*		*mg. per 100 gm*		*mg. per 100 gm.*	
Fed *vs.* fasted 24 hrs.	+5.7	0.01	−11.5	0.001	+5.4	0.001	+11.3	0.001	−1.1	0.1	+2.3	0.02	+0.9	0.1
Fasted 24 hrs. *vs.* fasted 48 hrs.	+9.1	0.001	+3.0	0.01	−0.3	0.7 −0.6	−1.7	0 05	+0.8	0.3	+1.9	0.01	+1.5	0.001

* p = percentage chance that a deviation as great or greater than that observed would arise by chance alone; any value of p 0.05 or less is usually accepted as indicating a significant difference.

fasting. The "mercuric-insoluble" P remained substantially unchanged throughout. The total acid-soluble P was decreased by about 15 per cent after fasts of 48 hours or longer. At that time the residual and the alcohol-soluble fractions were also diminished.

The data on the animals fasting 48 hours and on those fasting longer periods, because of their evident uniformity, were combined to yield the averages and standard errors summarized in the third row of Table I. The differences between the animals fasted for 24 hours and those fasted for

TABLE III

Distribution of Acid-Soluble Phosphorus in Livers of Rats Fasted Varying Periods of Time

Rat No.	Hrs. of fast	Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Phosphoglycerol P	"Mercuric-insoluble" P	Residual P	Alcohol-soluble P	Sum of fractions
		mg per 100 gm	*mg per 100 gm*	*mg per 100 gm.*	*mg per 100 gm*	*mg per 100 gm*	*mg per 100 gm.*	*mg per 100 gm*	*mg per 100 gm.*
G27	12	106.8	24.2	12.8	28.2	26.3	7.1	9.5	108.1
G28	12	110.2	23.2	14.5	27.0	29.0	4 6	6.7	105.0
G19	12	101.4	21.4	13.1	26 0		5.2	8.0	
G20	12	103.0	22.8	12.0	22.7		3.7	5.2	
G31	48	93.3	31.6	8.8	18.2	27.9	2.2	6.0	94.7
G32	48	90.9	31.7	8.1	18 0	30 7	1.4	3.4	93.3
G45	48	94.4	27.2	9.9	17.7	28.2	1.4	4 7	89.1
G46	48	88.7	25.7	10.0	16.5	28.0	0.9	4.5	85.6
G33	72	77.5	23.3	7.5	17.0	23.9	1.0	3.4	76.1
G34	72	92.7	25.4	11.9	22 4	28.1	−0.6	3.3	90.5
G47	72	85.9	28.3	11.3	17.3	28.0	1.6	4.0	90.5
G48	72	90.6	24.2	12.2	16 2	29 2	2.1	4.8	88.7
G35	96	88.5	26.9	13.1	18.1	25.9	2 8	3.6	90.4
G36	96	87.8	27.1	10.2	17.4	26.6	3.2	3.1	87.6
G57	96	85.5	26.0	9.6	18.6	27.2	1 4	4.1	86.9
G58	96	91.5	22.0	12.6	20.5	28.6	1.7	5.3	90.7
G59	96	91.6	22.1	12.9	18.8	29.7	1.7	5.7	90.9

48 hours or longer and the estimates of their significance are recorded in the second row of Table II. It can be seen that significant decreases of the inorganic, the residual, and of the alcohol-soluble P were found after the longer periods of fasting, in addition to the statistically very significant decrease of the total acid-soluble P. A slight increase of the phosphoglycerol is also suggested.

DISCUSSION

The significance of the changes in the distribution of phosphorus during fasting is as yet uncertain. In themselves they do not indicate either the direction or the extent of possible changes in the rates of turnover of the

phosphorus compounds in the liver. It seems likely that the new pattern in the distribution of phosphorus caused by fasting is correlated with some change in the metabolism of the body, probably with the shift from the combustion of carbohydrates to that of proteins and fats. This assumption is supported by the fact that the fasting levels of liver glycogen and the phosphorus changes occur at the same time.

The striking similarity between the changes of easily hydrolyzable and phosphoglycerol P is not explained by any of the current theories of the interrelationships between phosphorus compounds. Although these two fractions differ widely in their chemical stability, both appear labile *in vivo*. The changes of the inorganic P during fasting bear an obvious inverse relationship to them. Together, these three fractions form an apparently closely connected group. On the other hand, the fourth main fraction studied, the "mercuric-insoluble" P, appears unchanged during the fast. It is rather surprising that the changes of easily hydrolyzable P, representing the labile phosphorus of the nucleotides, are not reflected in changes in the "mercuric-insoluble" fraction, which contains the stable portion of the nucleotide P.[1] This would indicate that the concentration of the stable portion of nucleotides may remain constant, even though its degree of labile phosphorylation varies. This would mean that a changing mixture of nucleotides would be found in different conditions. That is, in fasting animals a larger proportion of the nucleotides would be present in the form of adenylic acid and adenosine diphosphate, and a smaller proportion in the form of triphosphate, as compared with fed animals.

The decrease of the total acid-soluble P concentration in the liver may perhaps be related to the acidosis of fasting. A much greater amount of phosphorus is lost owing to the decreased liver weight of fasted animals. Fasting for 24 hours reduced the liver weight from 9 gm., 4.6 per cent of body weight, to 5.5 gm., 2.8 per cent of the body weight. The loss of phosphorus amounts to over 4 mg. per 200 gm. of rat. This phosphorus may perhaps be taken up by the muscles, thus reversing the process occurring during glycogen deposition.

SUMMARY

In studies of the distribution of the acid-soluble phosphorus in the livers of fed and fasted rats, the concentration of inorganic P was found to be markedly increased after 24 hours of fasting, while that of the easily hydrolyzable and of the phosphoglycerol P was reduced considerably. The total acid-soluble P was reduced slightly after 24 hours of fasting and more after 48 to 96 hours.

[1] A few determinations of purine N also did not indicate any decrease of the nucleotide nitrogen during fasting.

BIBLIOGRAPHY

1. Nelson, N., Rapoport, S., Guest, G. M., and Mirsky, I. A., *J. Biol. Chem.*, **144**, 291 (1942).
2. Leva, E., and Rapoport, S., *J. Biol. Chem.*, **149**, 47 (1943).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
4. Booth, F. J., *Biochem. J.*, **29**, 2071 (1935).
5. Strack, E., Neubaur, E., and Geissendorfer, H., *Z. physiol. Chem.*, **220**, 217 (1933).
6. Fisher, R. A., Statistical methods for research workers, Edinburgh, 6th edition (1936).

THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE LIVERS OF FASTED RATS FED GLUCOSE, CASEIN, OLIVE OIL, OR A MIXED DIET

By S. RAPOPORT, ERNST LEVA, AND GEORGE MARTIN GUEST

(From the Children's Hospital Research Foundation and the Departments of Pediatrics and Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, April 5, 1943)

The marked changes in the distribution of the acid-soluble P in the liver of fasting rats (1, 2) suggested the possibility that the withdrawal of a single component of the diet was specifically responsible for these changes. To obtain further evidence on this point it was decided to study the ability of various substances fed to fasting rats to restore the distribution of phosphorus in their livers to normal. Accordingly, rats, after a preliminary fast of 48 hours, were divided into four groups, and were fed glucose, casein, olive oil, or a mixed diet. The values for the concentrations of total acid-soluble P and its four main fractions, namely the inorganic, the easily hydrolyzable, the phosphoglycerol, and the "mercuric-insoluble" P[1] in the livers of these rats, were determined and compared with values found in appropriate control groups.

Methods

Male rats weighing 155 to 245 gm. of Sprague-Dawley stock were used. They were kept on the stock diet of the laboratory (3) for at least 1 week before the beginning of the experiments. After a preliminary fast of 48 hours they were given access to weighed amounts of stock diet, glucose, casein, or olive oil for a period of 20 hours. The amounts consumed were determined by weighing the remaining food. Although some losses occurred through scattering of the food, they are believed to have been small compared with the amounts actually consumed. Throughout the experiments the animals had free access to drinking water.

During the preliminary 48 hour period of fasting the animals lost on the average 23.7 gm. of their body weight. During the feeding period of 20 hours they regained weight as follows: on the mixed diet, 15.1 ± 1.8 gm.; on glucose, 1.4 ± 0.9 gm.; on casein, 3.5 ± 0.7 gm.; and on olive oil, 1.5 ± 0.7 gm.

The preparation of the trichloroacetic acid extracts of the liver and the analytical methods used have been described previously (2, 4). The results were evaluated statistically according to the methods of Fisher (5).

[1] The fraction thus designated comprises phosphorus compounds which are precipitated with mercuric acetate at pH 5, as described under "Fractionation of extract" in the preceding paper.

TABLE I

Distribution of Acid-Soluble P in Livers of Rats Fed a Mixed Diet, Glucose, Casein, or Olive Oil

After a preliminary fast of 48 hours, the animals were offered the test foods for a period of 20 hours.

Test food	Rat No.	Food consumed	Liver weight	Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Phosphoglycerol P	"Mercuric-insoluble" P
		gm.	*gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*
Mixed diet	G37	14.5	9.3	90.7	19.6	19.4	22.9	
	G38	14.5	8.9	93.8	21.5	16.8	27.9	
	G49	13.0	8.1	85.7	18.0	15.0	25.4	25.6
	G50	12.5	9.4	88.8	16.7	15.2	24.9	26.1
	G72	15.0	9.2	100.2	22.8	12.1	25.3	29.0
	G75	10.0	7.6	95.6	16.2	17.7	25.0	28.5
	G76	10.0	7.0	93.4	16.1	17.1	23.7	29.1
		12.8	**8.5**	**92.6**	**18.7**	**16.2**	**25.0**	**27.7**
			±0.4	**±1.8**	**±1.0**	**±0.9**	**±0.6**	**±0.8**
Glucose	G39	8.5	8.9	82.0	15.7	13.3	22.0	
	G40	9.0	9.3	92.2	17.9	17.7	25.7	
	G51	9.0	9.8	85.5	15.4	17.4	22.5	24.3
	G52	8.3	8.7	83.8	16.0	16.3	25.1	26.0
	G73	8.1	7.2	111.8	20.6	18.8	22.6	22.6
	G74	9.0	7.9	100.4	15.0	20.2	25.0	25.0
	G77	9.7	7.7	98.0	13.5	20.5	26.8	26.8
	G78	10.0	8.8	93.8	10.1	22.9	26.5	26.5
		9.0	**8.5**	**93.4**	**15.5**	**18.4**	**24.5**	**25.2**
			±0.3	**±3.5**	**±1.1**	**±1.0**	**±0.7**	**±0.7**
Casein	G41	7.5	7.2	89.8	30.6	10.3	15.2	28.9
	G42	9.0	7.0	88.8	32.3	9.3	13.5	29.8
	G53	7.8	7.6	79.3	29.0	8.9	13.7	27.3
	G54	8.0	7.2	97.8	34.5	12.2	18.7	31.0
	G79	6.4	5.7	85.4	27.1	10.8	17.3	29.1
	G80	7.2	6.6	88.3	28.2	11.9	14.8	28.7
		7.7	**6.9**	**88.2**	**30.3**	**10.6**	**15.5**	**29.1**
			±0.3	**±2.5**	**±1.1**	**±0.5**	**±0.9**	**±0.5**
Olive oil	G43	5.0	5.0	91.2	26.1	7.7	19.2	27.9
	G44	5.0	5.0	91.1	25.3	9.2	16.1	28.1
	G55	4.6	6.4	87.0	23.8	8.0	17.6	26.0
	G56	4.6	5.4	86.0	24.5	10.0	17.3	27.2
	G81	5.1	4.8	92.3	26.9	9.3	21.0	28.1
	G82	6.8	5.1	88.0	23.0	13.3	20.2	26.8
		5.2	**5.3**	**89.3**	**24.9**	**9.6**	**18.6**	**27.4**
			±0.3	**±1.0**	**±0.6**	**±0.8**	**±0.8**	**±0.3**

The bold-faced figures indicate the mean values and their standard errors.

Results

In Table I are presented data on the effect of varying diets on the distribution of acid-soluble P in the livers of the rats. The individual data as well as the mean values for the total acid-soluble P and for four of its main fractions are included. In Table II are summarized the differences between the values for the four experimental groups and those for two control groups—one fed, and the other fasted for a period of 48 hours or longer. Also included in Table II are the estimates of the significance of these differences.

TABLE II

Acid-Soluble P in Livers of Rats. Differences and Estimates of Their Significance

		Total acid-soluble P		Inorganic P		Easily hydrolyzable P		Phospho-glycerol P		"Mercuric-insoluble" P	
		Difference	p*	Difference	p*	Difference	p*	Difference	p*	Difference	p*
		mg. per 100 gm		*mg per 100 gm*		*mg per 100 gm*		*mg per 100 gm*		*mg per 100 gm.*	
Fed *vs.*	Mixed diet	+11.3	**0.001**	−0.9	0.5	−0.4	0.7	+2.8	**0.02**	−0.2	0.9
	Glucose	+10.5	**0.02**	+2.3	0.1	−2.6	**0.05**	+3.2	**0.01**	+2.3	**0.01**
	Casein	+15.7	**0.001**	−12.5	**0.001**	+5.2	**0.001**	+12.2	**0.001**	−1.6	**0.05**
	Olive oil	+14.7	**0.001**	−7.1	**0.001**	+6.2	**0.001**	+9.2	**0.001**	+0.2	0.8
Fasted 48 hrs. or longer *vs.*	Mixed diet	−3.5	0.2	+7.6	**0.001**	−5.5	**0.001**	−6.8	**0.001**	+0.2	0.8
	Glucose	−4.3	0.3	+10.8	**0.001**	−7.7	**0.001**	−6.3	**0.001**	+2.7	**0.01**
	Casein	+0.9	0.8	−4.1	**0.01**	+0.1	0.9	+2.7	**0.02**	−1.3	0.1
	Olive oil	−0.1	0.9	+1.3	0.2	+1.1	0.3	−0.4	0.7	+0.5	0.3

* p = percentage chance that a deviation as great or greater than that observed would arise by chance alone; any value of p 0.05 or less is usually accepted as indicating a significant difference. The bold-faced figures indicate significant differences.

In the group fed the mixed diet the inorganic P and the easily hydrolyzable P were restored to the levels of the fed control group. The phosphoglycerol P, although greatly increased above fasting levels, was significantly lower than that in the fed controls. No significant changes were observed in the "mercuric-insoluble" P. The total acid-soluble P did not differ significantly from that in fasted rats.

In the glucose-fed rats the results in the main were similar but with some interesting modifications. The data suggest that the inorganic P was even lower, and the easily hydrolyzable P higher, than it was in the fed controls. As in the group receiving the mixed diet, the phosphoglycerol of this group was greatly increased, but remained significantly lower than that in the fed

rats. The "mercuric-insoluble" P of the glucose-fed rats was distinctly lower than that in either the fed or fasted control groups.

In the casein-fed rats the inorganic P was higher and the phosphoglycerol lower than in the fasted group, while the other fractions did not differ significantly.

Olive oil, of all the test foods, had the least effect on the distribution of the P in the liver, the concentrations of the various fractions remaining unchanged.

In Table I are also listed the liver weights of the various experimental groups. A comparison shows figures not significantly lower for rats fed a mixed diet or glucose than those for the fed controls. They were as low in the rats fed olive oil as in those fasted, and intermediate for the casein-fed group.

DISCUSSION

The data presented indicate clearly that glucose alone was able to produce the same effect on the distribution of phosphorus in the liver as a normal mixed diet. Consequently, one may assume that the changes of phosphorus found in fasted rats were due mainly to the withdrawal of carbohydrates. The lower concentrations of inorganic and the higher concentrations of easily hydrolyzable P of glucose-fed rats, compared with normally fed controls, suggest a relationship between these two fractions and the level of liver glycogen which is known to be above normal in such animals. The fact that the acid-soluble P did not increase, and that the phosphoglycerol level remained lower than in the fed controls, might indicate a relative deficiency of readily available P in the body. Nevertheless, the absolute amount of P in the liver was greatly increased, in direct proportion to the increased weight of the liver. This P, since none was fed to the rats, obviously was mobilized from other tissues, perhaps primarily from muscle. The "mercuric-insoluble" fraction, though somewhat diminished in concentration, was present in greatly increased amounts. This fact in conjunction with the behavior of the easily hydrolyzable P would indicate that the liver was able to synthesize with great ease the adenine nucleotides, represented in these fractions.

Owing to the higher inorganic P and the lower phosphoglycerol values, as compared with those of rats fasted 48 hours, the distribution of phosphorus in casein-fed rats was approximately the same as in those fasted for 24 hours only. In the two latter groups a preferential utilization of proteins may have constituted the common factor responsible for the similarity in the distribution of phosphorus. The failure of casein to restore the distribution of phosphorus to the levels of the fed rats merits attention in view of the fact that the livers of the casein-fed animals contained considerable

deposits of glycogen. The livers also contained increased amounts of P, in proportion to their increased weight. This would suggest a difference between the glycogen formation from proteins and from carbohydrates in their relationship to the phosphorus of the liver.

The following hypothesis may be advanced in explanation of the fact that the changes of metabolism are reflected by only moderate changes of the levels of the various P compounds. Some or all of the acid-soluble P compounds, though they may be chemically homogeneous, consist of two biologically differentiated parts, one nearly constant, concerned with the general metabolism of the liver cells, and the other variable, perhaps specifically related to the metabolism of carbohydrates. For instance, it might be assumed that a concentration of 16 mg. of phosphoglycerol per 100 gm. of liver, such as was found in fasted and casein-fed rats, is required for the normal function of the liver cells, while the increment of 12 mg. per 100 gm., found in fed rats, may have a specific function in their carbohydrate metabolism.

SUMMARY

In studies of the distribution of acid-soluble phosphorus in the livers of rats fed glucose, casein, olive oil, or a mixed diet after a preliminary fast of 48 hours, glucose alone was found capable of restoring the concentrations of inorganic, easily hydrolyzable, and phosphoglycerol P to the levels in the livers of fed control animals. The concentration of the total acid-soluble P of these rats remained at its fasting level.

BIBLIOGRAPHY

1. Nelson, N., Rapoport, S., Guest, G. M., and Mirsky, I. A., *J. Biol. Chem.*, **144**, 291 (1942).
2. Rapoport, S., Leva, E., and Guest, G. M., *J. Biol. Chem.*, **149**, 57 (1943).
3. Bills, C. E., Honeywell, E. M., Wirick, A. M., and Nussmeier, M., *J. Biol. Chem.*, **90**, 619 (1931).
4. Leva, E., and Rapoport, S., *J. Biol. Chem.*, **149**, 47 (1943).
5. Fisher, R. A., Statistical methods for research workers, Edinburgh, 6th edition (1936).

THE EQUILIBRIA OF ISOMERASE AND ALDOLASE, AND THE PROBLEM OF THE PHOSPHORYLATION OF GLYCERALDEHYDE PHOSPHATE*

BY O. MEYERHOF AND R. JUNOWICZ-KOCHOLATY

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, February 23, 1943)

As was shown some years ago (2–4) hexose diphosphate is split into triose phosphate by the reaction catalyzed by the enzyme zymohexase according to the following equation,

(1) Hexose diphosphate $\rightleftharpoons$ *d*-3-glyceraldehyde phosphate $\rightleftarrows$ dihydroxyacetone phosphate

While in the final state at least 95 per cent of the triose phosphate is dihydroxyacetone phosphate (2), it can be proved, nevertheless, that the reaction starts with the cleavage into 1 mole of aldotriose and 1 mole of ketotriose, owing to an enzyme which has been called aldolase. Isomerization is brought about by a second enzyme which has been called isomerase (5). The most direct proof of this sequence of reactions can be supplied by trapping the triose phosphate before isomerization sets in; *e.g.*, by hydrazine. In the presence of hydrazine about equal quantities of glyceraldehyde phosphate and dihydroxyacetone phosphate are obtained (6). The equilibrium constant of the isomerase reaction is of special interest, since in the steady state all sugar breakdown goes by the way of glyceraldehyde phosphate (7).

Negelein and Brömel (8) assumed that the precursor of the 1,3-diphosphoglyceric acid, which they had isolated, was a 1,3-diphosphoglyceraldehyde, formed spontaneously by addition of phosphate to 3-glyceraldehyde phosphate. If this is true, then in the presence of phosphate the following four equilibrium constants must exist.

$$\text{I.}\quad K_{\text{aldolase}} = \frac{[\text{3-glyceraldehyde phosphate}]\cdot[\text{dihydroxyacetone phosphate}]}{[\text{hexose diphosphate}]}$$

* A preliminary report of part of this paper was given at the Chicago meeting of the Federation of American Societies for Experimental Biology, April, 1941 (1).

Aided by grants from Hoffmann-La Roche, Inc., Nutley, New Jersey, and from the Penrose Fund of the American Philosophical Society.

$$\text{II.}\quad K_{\text{isomerase}} = \frac{[\text{dihydroxyacetone phosphate}]}{[\text{3-glyceraldehyde phosphate}]}$$

$$\text{III.}\quad K_{\text{zymohexase}} = K_{\text{isomerase}} \cdot K_{\text{aldolase}} = \frac{[\text{dihydroxyacetone phosphate}]^2}{[\text{hexose diphosphate}]}$$

$$\text{IV.}\quad K_{\text{phosphorylation}} = \frac{[\text{glyceraldehyde phosphate}] \cdot [\text{phosphate}]}{[\text{diphosphoglyceraldehyde}]}$$

Since part of the 3-glyceraldehyde phosphate would be removed by the formation of the hypothetical diester in the presence of inorganic phosphate, high concentrations of phosphate should act as an interceptor for the aldotriose. The measurement of the equilibrium of isomerase had been attempted previously (9) and no increase of the total aldotriose was found in the presence of inorganic phosphate (*cf.* (1)). Doubts, however, arose as to whether the previous value of 90 for $K_{\text{isomerase}}$ was correct. At equilibrium purified samples of triose phosphate, precipitated with barium acetate and alcohol, had been shown to contain 1.0 to 1.5 per cent glyceraldehyde phosphate. But owing to the instability of the triose phosphate, the purified samples correspond to only about one-fourth of the total triose phosphate formed in the enzymatic equilibrium and cannot be taken as representative, especially as the barium salt of the aldotriose is less soluble than that of the ketotriose (3).

These doubts were increased by the publication of a paper by Herbert, Gordon, Sabrahmanyan, and Green (10) on the purification of zymohexase. The authors studied the equilibrium of aldolase freed from isomerase ((I) above). Since equilibrium (III)[1] had been studied previously for various temperatures and concentrations of the reactants (2, 4, 5), $K_{\text{isomerase}}$ could now be calculated as $K_{\text{zymohexase}}/K_{\text{aldolase}}$. Using the aldolase values of Herbert *et al.* together with our own zymohexase values, one obtains $K_{\text{isomerase}}$ of about 25 to 30. Herbert *et al.* calculated with one value for $K_{\text{zymohexase}}$ that $K_{\text{isomerase}} = 36.3$. Although their presentation is confused by some numerical errors, the result as far as we can see is correct when their own experimental figures are used. In this way one obtains 2.7 to 4.0 per cent as the calculated content of the glyceraldehyde phosphate in the triose phosphate at equilibrium.

We therefore first redetermined the equilibrium constant of the isomerase reaction by improving the methods for measuring very small amounts of glyceraldehyde phosphate in the presence of large amounts of hexose di-

[1] In studying equilibrium (III) we took the concentration of dihydroxyacetone phosphate to be identical with the concentration of the total triose phosphate. The difference, owing to neglect of the small amount of glyceraldehyde phosphate, is negligible.

phosphate and dihydroxyacetone phosphate. After this was done, we applied the same procedure to equilibrium mixtures in the presence of high concentrations of inorganic phosphate, and of phosphate together with the purified oxidizing enzyme of Warburg and Christian (11, 12) in the presence and absence of cozymase, to find out whether equilibrium (IV) does exist and if so under what conditions.

Finally in the later stage of this work we repeated these measurements with purified isomerase which one of us (O. M.) had prepared with Dr. Lyle Beck. While the work on purification will be reported separately, some equilibrium experiments are included here to show the complete agreement with the K values obtained with isomerase in the presence of aldolase.

Preparation of Substrates and Enzymes—Hexose diphosphate, which was used for the measurement of equilibria as well as for the preparation of triose phosphate, was obtained by fermentation of glucose by dried brewers' yeast (Ballantine) and was purified as acid Ba salt by precipitation with alcohol at pH 3.8 according to suggestions of Robison.[2] In the preparation of a large amount of this salt, we had the kind help of Dr. Duschinsky of Messrs. Hoffmann-La Roche. The content of P in our preparation is 10 to 11 per cent, of Ba 36 per cent.

$C_6H_{12}O_{12}P_2Ba$.	Calculated.	P 13.05, Ba 28.9
$C_6H_{10}O_{12}P_2Ba_2$.	"	" 10.14, " 45.0

About a twelfth of the total P is inorganic. 90 to 94 per cent is saponifiable by alkali at 100° in 3 minutes (13); 84 to 90 per cent is transformable into triose phosphate in the presence of zymohexase and KCN and represents, therefore, the true content of hexose diphosphate of the preparation (14).

For *d-3-glyceraldehyde phosphate* we used triose phosphate prepared enzymatically in the presence of hydrazine (6). The principle of this method consists in capturing the triose phosphate during enzymatic incubation by means of an excess of neutralized hydrazine hydrochloride, decomposing the hydrazones in the deproteinized solution by means of benzaldehyde, and removing the benzaldehyde with ether. Fresh preparations of the barium salt contain a little more than 50 per cent *d*-glyceraldehyde phosphate and a little less than 50 per cent dihydroxyacetone phosphate. The dihydroxyacetone phosphate is less stable and decomposes slowly into methylglyoxal and phosphate, while the amount of *d*-glyceraldehyde phosphate is altered very little. In preparations kept some months in the ice box, more than 80 per cent of the remaining triose

[2] Herbert *et al.* refer to an unpublished method, recommended to them by the late Professor R. Robison. Probably this method is very similar to that used here (*cf.* MacLeod, M., and Robison, R., *Biochem. J.*, **27**, 286 (1933), foot-note p. 287).

phosphate consists of the latter. A fresh preparation contains 6.5 to 7.5 per cent triose phosphate P, 0.4 to 0.8 per cent inorganic P, and 0.5 to 0.7 per cent unsaponifiable organic P. When calculated from the organic P, it is 90 to 93 per cent pure triose phosphate. From 150 cc. of dialyzed muscle extract and 500 mg. of hexose diphosphate P in a total volume of 400 cc., 2.5 to 3.0 gm. of the purest fraction of the barium salt were prepared, containing more than 200 mg. of triose phosphate P.

Use of this preparation in experiments with *d*-glyceraldehyde phosphate has several advantages over the use of racemic glyceraldehyde phosphate (Fischer-Baer ester (15)). The barium salt is more stable, apparently being somewhat protected by the dihydroxyacetone phosphate, so that it can be used for several months with little decomposition of the glyceraldehyde ester. Since the dihydroxyacetone phosphate is optically inactive, the polarimetric molybdate method (16, 6) can be applied to measure the content of glyceraldehyde phosphate in the preparation itself as well as after enzymatic incubation. This method of high sensitivity is not applicable to racemic aldotriose. If isomerase is present, the total triose phosphate will be metabolized. Absence of isomerase in the enzyme fractions is tested by determining whether the percentage of glyceraldehyde phosphate in the total triose phosphate undergoes any change if incubated with the enzyme in question.

Cozymase, which was used for studying equilibrium (IV) in the presence of the oxidizing enzyme as well as for checking the activity of this enzyme, was prepared according to Ohlmeyer (17, 18), with some modifications introduced by Schlenk (19) and Jandorf (20).[3] In this way we prepared about 2 gm. of cozymase of 74 per cent purity. Smaller samples were purified with $AgNO_3$ + alcohol to a final purity of 85 per cent. The purest fractions were tested by hydrogen uptake in the presence of hydrosulfite (formation of dihydrocozymase (21)).

The following enzymes were prepared in various states of purity: zymohexase, aldolase, isomerase, and the "oxidizing enzyme" of Warburg and Christian. As *zymohexase* we used at first the dialyzed extract of an acetone powder of a fresh muscle extract. Since this extract contains impurities which impair the action of the oxidizing enzyme, we later employed a fraction obtained by partial saturation with $(NH_4)_2SO_4$, using a procedure similar to the first steps in the purification of the aldolase of Herbert *et al.* The fraction precipitating between 21 and 30 per cent ammonium sulfate was dissolved in 3 times its volume of distilled water, reprecipitated by 0.85 volume of a saturated ammonium sulfate solution containing one-twentieth

[3] We are much obliged to Messrs. Hoffmann-La Roche, Inc., for supplying us with 5 gm. of a crude fraction of cozymase (36 per cent pure).

of its volume of a 13 N NH_3 solution, and finally dialyzed for 20 hours against distilled water.

For obtaining *isomerase* free from aldolase we made use of the observation that it is more soluble in ammonium sulfate solution. The further purification will be described with Dr. L. Beck.

Aldolase free from isomerase was prepared according to Herbert *et al.*, but the last purification steps were omitted after it was observed that zymohexase B could be freed from isomerase by precipitation with 0.67 volume of a saturated ammonium sulfate solution, dissolving the precipitate in distilled water, and precipitating anew by ammonium sulfate brought to pH 5 with citrate. By this reagent aldolase is precipitated at a final ammonium sulfate concentration of 24.0 per cent (0.45 saturated), while isomerase remains in solution. The redissolved aldolase is adjusted to pH 8, since otherwise it is rapidly inactivated.

The *oxidizing enzyme* was prepared according to Warburg and Christian with some modification. In order to obtain a stable and nearly pure enzyme, of the same activity as that described by the German authors (1 γ of protein per cc., bringing the reduction of 0.5 mg. of cozymase to 80 per cent completion in 3 minutes at optimal pH and phosphate concentration), several changes have to be introduced in their procedure when maceration juice of American bakers' yeast is used as a source of the enzyme. The extract of the acetone powder is not heated to 55°, but immediately precipitated by acetic acid. The fraction obtained by neutralizing to pH 5.0 or 4.9 is discarded, and the fraction from pH 4.9 to 4.6 is kept. (Warburg and Christian discard the fraction until pH 4.75 is reached, but with our yeast most of the active enzyme would be lost.) The nucleoprotein, precipitated by acetic acid and redissolved at pH 6.0 with NaOH, is decomposed by salmine sulfate.[4] A solution of salmine sulfate containing 26 mg. per cc., warmed to 37°, is added to the solution of the enzyme until no further precipitation occurs. After treatment with $Al(OH)_3$ the solution is heated twice for 15 minutes at 58° to destroy aldolase and isomerase. If traces of the latter remain, the heating is repeated at 60°, with a negligible loss of activity. An essential modification of the method of Warburg and Christian consists in the addition of glutathione in neutral solution to a final concentration of M/40. Only in the presence of glutathione could the enzyme be kept in solution for weeks with little loss of activity. Glutathione apparently fulfils a double function: as complex former for traces of heavy metal salts and as reductant for the sulfhydryl groups of the enzyme (22). For all tests of the enzyme, glutathione was

[4] We are much obliged to Messrs. Sharp and Dohme, Inc., for their courtesy in supplying us with pure samples of salmine sulfate.

added to the dilutions so that its final concentration was at least 2.5×10^{-3} M. The activity of the oxidizing enzyme is tested manometrically with cozymase in the presence of arsenate, with $NaHCO_3$-NH_4HCO_3 mixture in an atmosphere of N_2 with 5 per cent CO_2. Two acid groups are generated by the following reaction,

(2) *d*-3-Glyceraldehyde phosphate + cozymase (+ arsenate) = *d*-3-phosphoglyceric acid + dihydrocozymase (+ arsenate)

Determination of Glyceraldehyde Phosphate—The outcome of the present work depends wholly on a reliable method of determining quantitatively very small amounts of glyceraldehyde phosphate. The principle of our former method consisted in the oxidation of the triose phosphate by iodine in weakly alkaline solution: the *d*-3-phosphoglyceric acid which is formed by oxidation is measured by its optical rotation in the presence of molybdate. Since $[\alpha]_D$ for *d*-3-phosphoglyceric acid is $-740°$ (16) the method is sensitive enough to be immediately applied to as little as 10 γ of P of glyceraldehyde phosphate per cc. of the original solution. But in the presence of interfering substances and with still greater dilutions it becomes necessary to concentrate the solutions and to remove or render harmless the interfering substances without destroying the glyceraldehyde phosphate. The interfering substances are phosphate, oxidized glutathione, and ammonium sulfate.

The former method of precipitation of the triose phosphate by barium acetate from the enzymatic mixture had to be abandoned because of the losses referred to above, especially since the fractionation in the presence of large amounts of inorganic phosphate leads to further losses. While inorganic phosphate up to 1.0 mg. of P per cc. (0.03 M) does not interfere with the polarimetric measurements, only slightly decreasing the value of the specific rotation, higher concentrations cause trouble in various ways and the phosphate must be removed.

Our new method consists in oxidizing the *d*-glyceraldehyde phosphate in the original trichloroacetic acid filtrate by iodine to *d*-3-phosphoglyceric acid, concentrating the solution *in vacuo*, removing the inorganic phosphate by magnesia mixture, and precipitating the phosphoglyceric acid together with the unchanged hexose diphosphate by barium acetate + alcohol. The advantage of this procedure is the immediate oxidation of the unstable glyceraldehyde phosphate to the very stable phosphoglyceric acid which withstands all manipulations without alteration. The main points involved may be described briefly.

Oxidation by Iodine—The glutathione in the enzymatic mixture interferes considerably with the oxidation of glyceraldehyde phosphate. Only by using a great excess of iodine, 1.5 to 2.0 cc. of M iodine + KI for 0.8

to 1 cc. of 0.1 N glutathione, and warming the solution during oxidation for half an hour at 37° does one succeed in destroying the oxidized glutathione and in bringing the oxidation of the glyceraldehyde ester to completion. This excess of iodine is also necessary to replace the iodine precipitated by ammonium sulfate in weakly alkaline solution. After completion of the oxidation the iodine is reduced by 30 per cent $KHSO_3$ in acid solution, and the iodoform which is formed by side reactions from methylglyoxal is removed by rapid centrifugation. The oxidized solution, neutralized to Congo red, is concentrated from an initial volume of 50 to 10 cc. in a Corning vacuum distillation apparatus at an outside temperature of 52°. This avoids the formation of decomposition products of hexose diphosphate, which can react with molybdate.

Inorganic phosphate is removed by ammoniacal magnesia mixture. The loss of phosphoglyceric acid by adsorption on the precipitate is small if a little less magnesia mixture is used than is sufficient for removing all of the inorganic phosphate.

After centrifuging, the cooled solution is neutralized to pH 6. Enough barium acetate is added to precipitate all sulfate and organic phosphate and the precipitation is completed by 2 volumes of alcohol. Such an amount of alcohol is necessary since the solubility of the acid barium salt of phosphoglyceric acid is increased in the presence of ammonium salts (23). The precipitate is centrifuged off, washed with 10 per cent alcohol, the alcohol removed by a current of air, and the precipitate vigorously shaken for 2 hours with 2 cc. of 2 N H_2SO_4. The solution after centrifuging plus the wash water of the precipitate is placed in a small measuring cylinder, neutralized, and cleared by rapid centrifugation. It amounts to 5 to 7 cc.

The *polarimetric measurements* are made in a Lippich-Landolt half shade polarimeter of 4 dm. length with monochromator. An 8 volt Mazda lamp with condenser served as the source of light. For the concentrated solutions semimicro tubes of 2 cc. capacity for 2 dm. or 4 cc. for 4 dm. were used.

The solutions were saturated with oxygen before the tubes were filled, in order to avoid a slow development of blue color with molybdate by the presence of traces of reducing substances. Generally 0.7 to 0.8 cc. of 30 per cent ammonium molybdate (Merck) was added to 2.0 cc. of the solution. The difference in rotation recalculated for this dilution before and after addition of molybdate is referred to in the headings of the tables as $\Delta\alpha$.

General Remarks—In many instances some of the steps of this procedure could be omitted; *e.g.*, the treatment with magnesia mixture if no phosphate was used in any sample of the experiment in question. The possible loss in the experiments of Tables I and V was checked by determining the

organic phosphate recovered in the solutions used in the polarimetric measurements. This content should be equal to the hexose diphosphate not transformed plus the phosphoglyceric acid generated by oxidation. The dihydroxyacetone phosphate is decomposed by the magnesia mixture and is determined as inorganic phosphate. Since the barium salt of phosphoglyceric acid is less soluble than that of hexosediphosphoric acid, the observed loss of organic phosphate, between 0 and 30 per cent, is somewhat higher than the loss of phosphoglyceric acid. Since it was necessary to have solutions concentrated enough for finding $\Delta\alpha$ values of at least 0.15° to 0.20°, losses as high as 30 per cent in unfavorable cases could not be avoided. On the other hand, blanks were run with the same enzyme mixture at zero time, and after incubation, but without oxidation. The latter is necessary in some cases, *e.g.* with addition of cozymase, to measure preformed phosphoglyceric acid in distinction to that formed by iodine. For the evaluation of the measurements without concentration, corrections for these blanks had to be applied. In the measurements of the concentrated barium precipitates the corrections were negligible and were not used in the final calculation of the results.

Determination of Phosphate—The various phosphate fractions are determined as in the preceding paper (18), by the Fiske-Subbarow method adapted to this purpose. The value found by the polarimetric method for glyceraldehyde phosphate is calculated as a percentage of the "alkali-saponifiable" phosphate; *i.e.*, the phosphate split off by NaOH in 15 minutes at room temperature. The amount of glyceraldehyde phosphate can also be measured by the decrease of this alkali-saponifiable phosphate produced by oxidation with iodine, whereby phosphoglyceric acid is formed. However, this method for the determination of glyceraldehyde phosphate by the difference of alkali-saponifiable P before and after iodine treatment, taken by Herbert *et al.* and Iri (24) from our older publications, is less accurate than the present method and has been used in the present work only for some preliminary experiments.

Equilibria in Absence of Inorganic Phosphate

While $K_{\text{zymohexase}}$ has been determined previously and K_{aldolase} had been measured by Herbert *et al.*, $K_{\text{isomerase}}$ is determined directly in the present paper. This was done in some cases with preparations of zymohexase, in others with purified isomerase. Since with the latter none of the triose phosphate is converted to hexose diphosphate regardless of temperature, larger readings can be obtained than with zymohexase provided that high concentrations of triose phosphate are used. Barium precipitation, therefore, is unnecessary.

We shall first describe the measurements with zymohexase. A series of preliminary experiments was run without concentrating; a 4 dm. tube

of 30 cc. capacity was used. The mean of ten such measurements between 60–20° was 4.2 per cent glyceraldehyde phosphate, with variations from 2.2 to 8 per cent. Since the rotations were about $-0.10°$ and corrections for impurities had to be applied, the results did not prove to be accurate enough. In Table I all experiments carried out according to the procedure described in the foregoing section are reproduced. Since they were performed in the absence of inorganic phosphate, Mg precipitation was omitted. The twenty-five experiments at 60–30° give an average value of 4.4 per cent glyceraldehyde phosphate in the total triose phosphate, independent of temperature; the means of 4.05, 4.8, and 4.2 for 60°, 40–38°, and 30° respectively agree within the limits of accuracy of the method. The losses by incomplete oxidation and incomplete recovery of the barium precipitate may amount to 10 per cent or somewhat more. The experiments of Table I are evaluated for the equilibrium constants in Table II.

The marked variation of the equilibrium of zymohexase with temperature, which obeys closely the van't Hoff law of isochores (2, 4), is wholly due to the aldolase, as had been deduced previously from the thermodynamics of the reaction. There is a dependence on pH too, to which less attention was paid in former work. The K values are maximal in the neighborhood of pH 7.5 and generally lower at pH 8.5 and 7.0. Since most of the experiments of Table I were performed in the absence of buffers, but near to neutrality, differences of $K_{\text{zymohexase}}$ for the same temperature are mainly due to slight variations of the pH. This influence is due to the aldolase, since the isomerase is not affected by the pH.

The results obtained for the different equilibrium constants can be checked by two independent sets of experiments: determination of the equilibrium with aldolase preparations free from isomerase and with isomerase free from aldolase. The first has already been done in part by Herbert *et al.* But since they had made no measurements at 60°, at which our values are the most accurate, we carried out such a series of experiments, reproduced in Table III. For thirteen different preparations of aldolase made by various methods of fractionation of zymohexase, all free of isomerase, the K_{aldolase} at 60° was determined according to the equation $K_{\text{aldolase}} = \frac{1}{4} \times [\text{triose phosphate}]^2/[\text{hexose diphosphate}]$. The absence of isomerase is assumed, if by iodine oxidation 50 per cent or more of the alkali-saponifiable P has disappeared. (The disappearance is a little greater than 50 per cent, because in addition to the glyceraldehyde phosphate some of the dihydroxyacetone phosphate is destroyed by the alkalinity during oxidation.) The mean value for K_{aldolase} at 60° in Table III amounts to 4.0×10^{-4} instead of 4.5×10^{-4} shown in Table II. For 38° Herbert *et al.* found with purified aldolase 1.19×10^{-4}, which corresponds closely to the value 1.33×10^{-4} found in Table II for the same temperature.

A similar agreement results from the experiments with isomerase freed

TABLE I

Per Cent of Glyceraldehyde Phosphate in Zymohexase Equilibrium

Temperature	Experiment No.	Protocol No.	Enzyme preparation	Time	Volume*	Hexose diphosphate†	Triose phosphate formed	$-\Delta\alpha$‡	Glyceraldehyde phosphate	Glyceraldehyde phosphate in triose phosphate	Mean	Recovery of P§
°C.				sec	cc	mg. P	per cent	degrees	mg. P	per cent	per cent	per cent
60	1	55	Ac.	40	65	40.4	40	0.915	0.66	4.1		
	2	55	"	80	65	40.4	35	0.56	0.39	2.8		
	3	59	"	60	71	28.3	52	0.804	0.51	3.45		
	4	65	"	50	63	30	47	0.654	0.535	3.75		
	5	166	"	55	55.5	11.5	57	0.306	0.17	2.9		
	6	167	"	50	56.5	10.5	60	0.503	0.34	5.4		
	7	167	"	80	56.5	10.5	58.5	0.402	0.255	4.15		
	8	167	"	120	56.5	10.5	59.5	0.417	0.275	4.4		
	9	169	Z. B.	45	56.5	11.0	57	0.475	0.30	4.8		
	10	169	"	80	56.5	11.0	63	0.465	0.295	4.2		
	11	175	"	90	56.5	10.2	64 5	0.462	0.31	4.7		95
	12	217	Z. di.	75	34.2	14.0	44.5	0.43	0.35	4.0	4.05	100
40	13	206	Z. B.	480	64	24.7	27	0 365	0.44	7.4		90
	14	222	Z. di.	600	44	15.3	26	0.193	0.173	4.2		98
	15	222	½ Z. di.	600	44	15.3	23.4	0.198	0.162	4.5		83
	16	226	Z. di.	480	46.6	15.1	36.7	0.175	0.173	3.15		
38	17	169	Z. B.	600	58	11.0	36.4	0.245	0.143	3.6		
	18	175	"	480	56.5	10.2	38.5	0.27	0.167	4.25		94
	19	175	"	1080	56.5	10.2	39	0.43	0.25	6.3		97
	20	175	Ac.	360	56.5	10.2	41	0.29	0.18	4.3		96
	21	175	"	720	56.5	10.2	42	0.39	0.24	5.6	4.8	90
30	22	54	"	600	34	20.2	18.3	0.264	0.185	5.0		100
	23	217	Z. di.	780	34.2	14.1	18.6	0.17	0.11	4.2		100
	24	227	"	900	37.6	14.0	25.5	0.095	0.10	2.8		92
	25	227	"	900	37.6	14.0	22	0.13	0.15	4.9	4.2	100

Experiments with the same protocol number were carried out with the same enzyme preparation but with different amounts or at different time intervals.

Ac. = dialyzed extract of acetone powder; Z. B. = preparation of zymohexase B. Z. di. = the same, dialyzed to remove the ammonium sulfate.

* Volume includes 3 to 5 cc. of 40 per cent trichloroacetic acid; this amount is deducted in Table II for calculation of the molar concentrations.

† Only the amount transformable into triose phosphate = 84 to 92 per cent of the organic phosphate of the preparation is accounted for.

‡ Rotation difference with and without molybdate, measured in a 2 dm. tube for concentrated samples of 4 to 7 cc., corresponding to 80 to 90 per cent of the initial volume (sixth column).

§ The initial hexose diphosphate minus the dihydroxyacetone phosphate formed is taken as 100 per cent.

from aldolase. In order to have polarimetric readings, after establishment of the equilibrium, of as much as $-0.15°$ to $-0.20°$ with molybdate in 2 dm. tubes, nearly 1 mg. of P of triose phosphate per cc. is required in the original solution. To such solutions, buffered with borate at pH 7.6, various dilutions of isomerase were added. In Table IV only those experiments are reproduced in which it was ascertained, by incubation during

TABLE II

Equilibrium Constants Calculated from Experiments of Table I

Temperature	Experiment No.	Hexose diphosphate		Triose phosphate	Glyceraldehyde phosphate	Dihydroxyacetone phosphate	$K_{zymohexase}$	$K_{isomerase}$	$K_{aldolase}$	$K_{aldolase}$, mean
		Initial	Final							
°C.		$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	10^{-3}		10^{-4}	10^{-4}
60	1	10.5	6.3	8 4	0.345	8.06	10.3	23.4	4.4	
	2	10.5	6.55	7.85	0 22	7.63	8 7	34.2	2.55	
	3	6.9	3.35	7.1	0.245	6 85	14.0	28	5.0	
	4	8.1	4.3	7.6	0.285	7.31	12 4	25.7	4.8	
	5	3.34	1.56	3.56	0.104	3.45	7.55	32 8	2 3	
	6	3.16	1.25	3.82	0.202	3.61	10.4	17.4	6.0	
	7	3.16	1.305	3 71	0 154	3.55	9.6	23.0	4.18	
	8	3.16	1.285	3.78	0 165	3 58	10.2	23 2	4.6	
	9	3.31	1.40	3.82	0.163	3 56	9.0	21 8	4.15	
	10	3.31	1.20	4.22	0.178	4 04	13.2	22.8	6.0	
	11	3.06	1.06	4.00	0.188	3.81	13 5	20 2	6 7	
	12	7.0	3.86	6.28	0.25	6.03	9 4	24	3.9	4.5 ($K_{60°}$)
40	13	6.65	5 06	3.18	0.235	2 95	1.71	12 5	1.37	
	14	5.87	4.30	3.15	0 132	3.02	2.15	23	0.93	
	15	5.87	4.47	2.8	0.126	2.67	1.61	21 5	0.75	
	16	5 74	3.66	4.17	0.131	4 04	3 95	31	1.27	
38	17	3.31	2.15	2.32	0.083	2.24	2 4	27 4	0.87	
	18	3.06	1.88	2.37	0.103	2.27	2.75	22.0	1.25	
	19	3.06	1 80	3.08	0.158	2.35	3.08	15 0	2 05	
	20	3.06	1.80	2.53	0.109	2.42	3.26	22.2	1 47	
	21	3.06	1.72	3.35	0.144	2 43	3 35	17 0	1.98	1.33 ($K_{38-40°}$)
30	22	10.5	8.6	3 84	0.192	3.65	1.41	19 0	0.74	
	23	7.05	5.73	2 63	0.11	2 52	1.12	23	0 485	
	24	6 53	3 21	3 32	0.093	3.23	3 20	34.5	0 93	
	25	6.53	3.53	3.0	0.147	2.85	2.07	19.5	1.06	0.80 ($K_{30°}$)

different intervals or with different dilutions of the enzyme, that the final end-point was practically reached. The mean value of twelve such experiments, given in Table IV, is 4.3 per cent glyceraldehyde phosphate in the total triose phosphate. The measurements could not be made at 60°, because too much triose phosphate was decomposed during the heating time.

Equilibria in Presence of Phosphate

The considerable effort spent in ascertaining the numerical values of the aldolase and isomerase equilibria was in large part intended as a preliminary for settling the question whether phosphate has any influence on these equilibria.

Negelein and Brömel (8), announcing their discovery of the 1,3-diphosphoglyceric acid, write, "If one adds inorganic phosphate to glyceraldehyde-3-phosphoric acid (Fischer ester), 1 mole of phosphate is taken up in the reversible reaction: phosphate + glyceraldehyde phosphate $\rightleftharpoons$ diphosphoglyceraldehyde." This is only a hypothesis for interpreting the reversible formation and reduction of diphosphoglyceric acid in the presence of the oxidizing enzyme, cozymase, phosphate, and glyceraldehyde phosphate. How may such an issue be decided experimentally? We must distinguish between two possibilities. The formation could be a mere physicochemical equilibrium, as Negelein and Brömel believe, or an enzymatic reaction. In the latter case, since only the crystallized enzyme of Warburg and Christian is present, this would be at the same time an oxidizing and phosphorylating enzyme. In either case the possibility of

TABLE III

Aldolase Equilibrium at 60°

Protocol No.	Ammonium sulfate fraction of aldolase	Time	Hexose diphosphate, initial	Triose phosphate formed	Triose phosphate	Triose phosphate removed by I_2	$K_{aldolase}$
		sec	$M \times 10^{-3}$	*per cent*	$M \times 10^{-3}$	*per cent*	10^{-4}
22	B	80	12.9	17.8	4.6	55	4.95
	B_2	30	12.9	16.5	4.26	60	4.2
	"	80	12.9	18.2	4.7	57	5.0
	B dialyzed	80	12.9	17.8	4.6	55	4.95
23	"	90	13.05	17.5	4.53	64	4.35
	B_e	60	13.05	19.0	4.8	50.5	5.4
	B_{e_a}	30	13.05	15.5	4.0	66	3.6
	"	90	13.05	16.4	4.25	69	4.1
24	B	30	13.2	16.0	4.23	67	4.05
	B_e	30	13.2	16.6	4.40	50	4.4
34	C_g	60	11.85	15.2	3.62	50	3.3
	C_{g1}	30	11.85	14.0	3.32	56	2.7
	C_{g2}	30	11.85	13.3	3.17	51	2.45
35	G_3	40	11.85	13.5	3.21	55	2.55
	"	90	11.85	14.4	3.43	50	2.9
36	G_6	30	11.1	17.6	3.93	58.5	4.2
	"	90	11.1	17.9	4.0	50	4.4
Mean							4.0

demonstrating such an intermediary would depend on the equilibrium concentration; that is, of the value of $K_{\text{phosphorylation}}$ in equilibrium (IV). Warburg and Christian published three curves showing the dependence of the reduction of cozymase upon phosphate concentration. 33×10^{-3} M phosphate had about the maximal effect, while 16×10^{-3} M gave 84 per cent and 0.87×10^{-3} M 25 per cent of this value. With Dr. Drabkin, we made many more determinations with different concentrations of phosphate and triose phosphate. If a simple diphosphoglyceraldehyde should exist, the reduction of cozymase must obey the equation

TABLE IV

Equilibrium with Purified Isomerase in Absence of Phosphate

Protocol No.	Dilution of enzyme	Temperature	Time	Triose phosphate per cc.	Polarimeter tube	$-\Delta\alpha$ measured	Glyceraldehyde phosphate per cc., initial solution	Glyceraldehyde phosphate in triose phosphate
		°C.	*min*	*mg P*	*dm.*	*degrees*	*mg. P*	*per cent*
232	1:30	37	12	0.48	4	0.268	0.024	5.0
	1:90	37	12	0.48	4	0.250	0.021	4.4
	1:90	37	21	0.49	4	0.22	0.018	3.7
244			0	1.215	2	5.62	0.79	65
	1:500	38	8	1.19	2	0.36	0.059	4.95
	1:500	38	15	1.16	2	0.343	0.055	4.75
246			0	0.90	2	3.87	0.472	52.5
	1:40	21	15	0.84	2	0.167	0.027	3.5
	1:40	21	30	0.84	2	0.187	0.033	3.9
	1:40	21	60	0.64	2	0.15	0.027	4.25
	1:40	31	15	0.74	2	0.181	0.031	4.2
247			0	0 82	2	3.54	0.562	69
	1:60	25	16	0.794	2	0.206	0.032	4.0
261			0	0.560	2	2.24	0.408	72.8
	1:2200	22	40	0.540	2	0.131	0.0241	4.3
	1:45	22	40	0.570	2	0.125	0.0234	4.2
Mean of final equilibrium								4.3

$$K_{\text{reduction}} = \frac{[\text{diphosphoglyceraldehyde}] \cdot [\text{cozymase (oxidized)}]}{[\text{diphosphoglyceric acid}] \cdot [\text{dihydrocozymase}]}$$

Since in the isolated system [dihydrocozymase] = [diphosphoglyceric acid], the above equation can be written

$$K_{\text{reduction}} = \frac{[\text{diphosphoglyceraldehyde}] \cdot [\text{cozymase (oxidized)}]}{[\text{dihydrocozymase}]^2}$$

and multiplied by $K_{\text{phosphorylation}}$

$$K'_{\text{reduction}} = \frac{[\text{glyceraldehyde monophosphate}] \cdot [\text{phosphate}] \cdot [\text{cozymase (oxidized)}]}{[\text{dihydrocozymase}]^2}$$

Actually the value for $K'_{\text{reduction}}$ decreases with increasing concentrations of phosphate, while it increases with increasing concentrations of glyceraldehyde phosphate and constant high phosphate concentrations. Whatever interpretations are offered for these discrepancies, $K_{\text{phosphorylation}}$ could not be greater than approximately 10^{-2}, since otherwise the strong effect on reduction of cozymase by phosphate concentrations of 10^{-2} to 10^{-3} would be impossible to explain.[5]

Let us now assume the first of the above possibilities, that the equilibrium of phosphorylation is non-enzymatic. Then with the value of $K_{\text{phosphorylation}}$ of 10^{-2} the amount of diphosphoglyceraldehyde can be calculated for all concentrations of phosphate. For 0.1 M inorganic phosphate the proportion of diphospho ester to monophospho ester would be 10. In the presence of zymohexase or isomerase the equilibrium would be immediately affected. With a value of $K_{\text{isomerase}} = 22$, triose phosphate would be distributed in the presence of 0.1 M phosphate into 67 per cent dihydroxyacetone phosphate, 3 per cent glyceraldehyde monophosphate, and 30 per cent diphosphate, instead of 96 per cent dihydroxyacetone phosphate and 4 per cent glyceraldehyde phosphate in the absence of phosphate. By destroying the zymohexase with trichloroacetic acid, dilution and oxidation by iodine of the glyceraldehyde monophosphate, the diester would dissociate into its components. This would be quite the same if the decomposition were not really brought about by removing the monoester, but by the chemical instability of the diester in the absence of the zymohexase system, by which it would be continuously regenerated.

The situation would be somewhat different if the presence of the oxidizing enzyme with or without cozymase were necessary for the formation of the diester. Since phosphate concentrations higher than 6×10^{-2} M do not continue to increase the equilibrium concentration of diphosphoglyceric acid, this could be true for the phosphate uptake too, and might depend on a sensitivity of the enzyme toward the higher concentrations. One could expect the formation of the diester in this case only under the same conditions in which the formation of dihydrocozymase takes place. Moreover, in destroying the enzyme the diester could in principle be pre-

[5] In the presence of 1.5×10^{-3} M inorganic phosphate 15 to 20 per cent of the maximal reduction of cozymase is obtained; in the presence of 10×10^{-3} M 50 per cent. With $K_{\text{phosphorylation}} = 10^{-2}$ in the first case less than 12 per cent and in the second case about 50 per cent of the total glyceraldehyde phosphate would exist as diester. If $K_{\text{phosphorylation}}$ is assumed to be 5×10^{-2}, the respective concentrations of the diester would be less than 3 and 16 per cent, which would be less compatible with the effect on the reduction of cozymase by these concentrations of phosphate.

served even in the absence of the monoester, although it is not very likely that it would withstand the oxidation by iodine and would decompose in any other way than via glyceraldehyde monophosphate.

Because of this, it is necessary before the possible enzymatic formation of the diphosphoglyceraldehyde is studied to check the preparations of the different enzymes for their ability to reduce cozymase in dependence on the phosphate concentration and at similar temperatures, times, etc., as can be used for testing the existence of an equilibrium of phosphorylation.

Generally the oxidizing enzyme was at first tested for reaction (2) above in the presence of arsenate, by the manometric technique. Enzyme dilutions were used which brought this reaction to completion at 20° in about 20 minutes with 0.6 to 0.8 mg. of pure cozymase and an excess of glyceraldehyde phosphate. In the same test the absence of aldolase in the oxidizing enzyme was established by showing that triose phosphate cannot be replaced by hexose diphosphate. The dilution of zymohexase necessary for inducing hexose diphosphate to react with nearly the same speed as triose phosphate was also determined in this way. Finally the absence of isomerase in the oxidizing enzyme was tested by the polarimetric molybdate method.

These experiments served as the basis for the incubation of hexose diphosphate in the presence of zymohexase, phosphate, oxidizing enzyme with and without cozymase, and for the subsequent determination of glyceraldehyde phosphate by oxidation with iodine.

When cozymase is present, a small amount of diphosphoglyceric acid is formed, equivalent to the amount of dihydrocozymase. This amount is determined in the same fraction as the glyceraldehyde phosphate in the form of 3-phosphoglyceric acid. However, since the amount of cozymase used is lower than the concentration of glyceraldehyde phosphate at equilibrium, the diphosphoglyceric acid corresponds only to one-tenth of the latter, which is within the error of measurement. With impure preparations of enzymes the reaction could proceed further by formation of 3-phosphoglyceric acid. Such a course is easily detected, by the optical rotation in molybdate without oxidation by iodine. Indeed if any phosphoglyceric acid was formed in experiments in the presence of cozymase (besides the minute amount of the diester), it was taken as a sign that the enzymes were not sufficiently purified and the purification was continued.

The zymohexase and isomerase equilibria were in no instance changed by the presence of inorganic phosphate, either directly or in the presence of the oxidizing enzyme alone or with addition of cozymase. The accuracy is slightly less than in the absence of phosphate, owing to the cumbersome method of isolation of the phosphoglyceric acid. Moreover, the total triose phosphate cannot be accurately determined in the presence of high con-

centrations of phosphate. Sometimes it was determined by methylglyoxal formation after acid hydrolysis (2) instead of by saponification with alkali. The results showed that up to 0.1 M phosphate, the amount of total triose phosphate was not appreciably altered at equilibrium. In the routine experiments, therefore, one sample was incubated with the same additions except inorganic phosphate, and the alkali-labile P found here was assumed to be present also in the other samples.

That the first alternative, a non-enzymatic equilibrium of phosphorylation, can be excluded was indicated by direct polarimetric readings in molybdate after incubation of hexose diphosphate with zymohexase in the presence of 0.1 M phosphate. Although this method is not sufficiently accurate for determining the exact equilibrium concentration of glyceraldehyde phosphate, a nearly 10-fold increase, corresponding to $K_{\text{phosphorylation}} = 10^{-2}$, would give rotations in the 4 dm. tube of more than $-1°$ for equilibria at 60° and more than $-0.5°$ at 30°. Such rotations were completely absent. Instead of this, six such experiments, four at 60°, one at 30°, and one at 20°, gave on the average an equilibrium content of glyceraldehyde phosphate of about 4 per cent (between 2.4 and 8.1 per cent), the same as in the absence of phosphate.

The more accurate determinations after concentration and barium precipitation were, therefore, immediately employed for the study of this system in the presence and absence of the oxidizing enzyme and cozymase. Since such an enzymatic equilibrium is possibly dependent on temperature and since our absorption measurements of dihydrocozymase which served for reference had to be made at room temperature, it seemed advisable to use relatively low temperatures of incubation. Since the zymohexase equilibrium is too unfavorable at 20°, these experiments are done at 30° and 40°, while with isolated isomerase temperatures of 22° and 25° can be used. Table V contains the data of the most reliable experiments. For comparison some data (Experiments 1a and 8a) are included from experiments in which the enzymatic incubation was made in the absence of phosphate (but phosphate was added after deproteinization) and in which the treatment with Mg mixture, etc., was identical with that of the other sample under the same number. The conditions of the experiments were identical with those of Table I except for the addition of phosphate, oxidizing enzyme, and cozymase and the removal of the inorganic phosphate by magnesia mixture. 0.1 to 0.5 cc. of the stock solution of oxidizing enzyme (E of Table V), containing 0.2 to 0.5 mg. of protein, was used in a total volume of about 40 cc., and in some experiments 7.5 mg. of cozymase of a purity of 0.74 were added. The concentration of the protein was somewhat higher than in the absorption measurements.

The last column of Table V shows that in a few cases losses of the total organic phosphate of about 30 per cent occur, but generally they are much

less. The loss of phosphoglyceric acid is smaller than the total loss. On the other hand, the slight increase over 5 per cent glyceraldehyde phosphate in some cases with oxidizing enzyme cannot be regarded as significant, since similar variations occur in Table I in the absence of phosphate.

Some further experiments were made with purified isomerase at 38°, 25°, and 22°. Here any kind of pretreatment can be avoided. As is shown in Table VI, the glyceraldehyde phosphate content at equilibrium is the same as in the absence of phosphate and the oxidizing enzyme. This is still

TABLE V

Per Cent of Glyceraldehyde Phosphate in Presence of Zymohexase, Oxidizing Enzyme, and Phosphate

Temperature	Experiment No.	Protocol No.	Phosphate	Enzyme*	Time	Volume	Hexose diphosphate	Triose phosphate	$-\Delta\alpha$ (2 dm. tube)	Glyceraldehyde phosphate	Glyceraldehyde phosphate in triose phosphate	Recovery of organic P
°C			10^{-2}		min	cc	mg P	per cent	degrees	mg. P	per cent	per cent
60	1	217	4.7		1.25	34.2	14.0	44 5	0.362	0.254	4.05	88
	1a	217	0		1.25	34.2	14 0	44.5	0.337	0.260	(4.15)	100
40	2	206	5.0		8	32	12.35	27	0.123	0.109	3.7	73
	3	206	5.0	E	8	64	25 7	27	0.266	0.406	6.85	66
	4	222	3.6	"	10	44	15.6	26	0.207	0.182	4.4	95
	5	222	3.6	" + CO	10	44	15.6	26	0.270	0.26	6.3	84
	6	222	3.6	½ E + CO	10	44	15.6	26	0 243	0 30	7.3	100
30	7	217	4.7		13	34.2	14.0	18.6	0 105	0.073	2.8	86
	8	217	4.7	E + CO	13	68.4	28.0	18.6	0.224	0.208	4 0	92
	8a	217	0		13	34.2	14.0	18.6	0.144	0.108	(4 1)	96
	9	227	4.6	E	15	34 2	14.0	21.9	0.140	0.152	4 95	75
	10	227	4.6	" + CO (pH 7.0)	15	34.2	14.0	25 5	0.134	0.138	3.9	85
	11	227	4.6	" + CO (pH 8.0)	15	34.2	14 0	24.9	0.144	0.150	4.8	94

* E = oxidizing enzyme; CO = cozymase.

more conclusive evidence, since any interference with the activity of the isomerase by the additions would make the concentration of glyceraldehyde phosphate too high but never too low.

Further negative results were obtained with direct polarimetric measurements of the glyceraldehyde phosphate in the presence of phosphate and the oxidizing enzyme. A diphosphoglyceraldehyde must contain 2 asymmetric carbon atoms. If the phosphate uptake into the carbonyl group takes place by enzymatic action, the formation of only one of the two stereoisomeric forms is to be expected. A great difference in optical rotation should

result compared with the glyceraldehyde monophosphate. Glucose-1-monophosphate (Cori ester), having such a phosphorylated carbonyl group has $[\alpha]_D = +120°$, which corresponds to $+174°$ for the molecular weight of glucose, while α-glucose has $[\alpha]_D = +105°$ (25). This phosphorylation creates, therefore, a difference of $[\alpha]_D = +69°$ in the specific rotation of glucose.

In our case no difference was found which exceeded the accuracy of the measurements. $[\alpha]_D$ of *d*-3-glyceraldehyde phosphate was determined some years ago and found to be $+20°$ in slightly acid solution (6). The rotation is greatly influenced by the pH. In our present measurements we find at pH 5 $[\alpha]_D = +19.5°$ to $+22°$, at pH 6 $+17°$, and at pH 7 $+12°$.

TABLE VI

Equilibrium of Isomerase in Presence of Phosphate and Oxidizing Enzyme

Protocol No	Temperature	Time	Phosphate	Enzyme	Triose phosphate per cc	$-\Delta\alpha$ (2 dm tube)	Glyceraldehyde phosphate per cc. initial solution	Glyceraldehyde phosphate in triose phosphate
	°C.	*min.*	M × 10^{-2}		*mg. P*	*degrees*	*mg. P*	*per cent*
232a	38	12	5.0		0.60	0.75	0.0243	4.0
	38	12	5.0	E	0.60	0.16	0.027	4.5
	38	12	0		0.60	0.19	0.033	(5.5)
235	38	12	6.0		0.79	0.29	0.045	5.7*
	38	12	6.0	E	0.79	0.306	0.0465	5.9*
247	25	16	5.5		0.794	0.22	0.0335	4.2
	25	16	4.4	E	0.794	0.213	0.0306	3.85
	25	16	4.4	" + CO	0.794	0.266	0.040	5.0
261	22	40	5.9		0.560	0.097	0.0177	3.15
	22	40	4.0	E	0.560	0.103	0.0197	3.5
	22	40	0		0.570	0.125	0.0234	(4.2)

* Equilibrium not quite complete.

A 0.75 per cent solution (calculated for glyceraldehyde phosphoric acid) gives at pH 7 in a 2 dm. tube $\alpha = +0.18°$, and when dissolved in 0.2 M phosphate of pH 7.0, $+0.185°$. When oxidizing enzyme is added, corrections must be made for glutathione contained in our enzyme solutions. A solution of 0.97 per cent glyceraldehyde phosphate in 0.064 M phosphate at pH 6 gives $\alpha = +0.33°$, corresponding to $[\alpha]_D = +17°$, while with oxidizing enzyme the rotation of an exactly equivalent sample after correction is $\alpha = +0.31°$.

DISCUSSION

Since no dissociable diphosphoglyceraldehyde could be detected under conditions in which cozymase is reduced and diphosphoglyceric acid is

formed, we may briefly discuss what possibilities remain for a precursor substance. One such possibility would be that the precursor formed enzymatically would be undissociable in the absence of the enzyme, like the dimeric diphosphoglyceraldehyde of Baer and Fischer (26). This substance itself proved to be biologically inactive ((18) foot-note 1). But it is a single racemic body whose biochemical breakdown was improbable from the start. A similar substance formed by two *d*-glyceraldehyde groups could perhaps react. But such a possibility may be practically ruled out by the following observation. As was found by us and reported in the paper of Baer and Fischer, the racemic diphosphoglyceraldehyde is split by 5×10^{-2} HCl at 100° in 8 minutes rather completely into monomeric glyceraldehyde monophosphate and inorganic phosphate. The same result obtains in the presence of phosphate by addition of HCl until pH 1.5 is reached. Such a treatment applied to deproteinized isomerase mixtures in the state of equilibrium in the presence of phosphate and oxidizing enzyme, like those of Table VI, does not increase the amount of glyceraldehyde phosphate.

The opposite explanation, therefore, seems likely: that the product is too labile to be detected. To be sure, the instability has been viewed throughout this work as the special feature by which the product could be detected provided that it decomposes into glyceraldehyde phosphate and phosphate. Aside from the rather remote possibility that it breaks down instantaneously in another way, one has to visualize the formation of a physical addition product so loose that it would behave towards isomerase not as a compound, but like free glyceraldehyde phosphate, and not shift the equilibrium at all. In this case dihydroxyacetone phosphate would probably form a similar addition product. But this latter assumption would not by itself explain the experiments with zymohexase in the presence of phosphate, since an equal phosphate uptake by both triose phosphates while neutral to isomerase should affect the aldolase equilibrium by increasing the proportion of triose phosphate to hexose diphosphate. This does not happen: the methylglyoxal split by acid hydrolysis is not increased in the presence of phosphate. The total triose phosphate, therefore, and not only the aldose part is unchanged.

Several known facts can be quoted in favor of the idea that only a physical addition product is formed, which, nevertheless, makes the carbonyl group more unstable to oxidizing agents. As was shown by Warburg and Yabusoe (27) and Wind (28), ketohexoses (fructose, sorbose) and trioses (dihydroxyacetone and glyceraldehyde) are oxidized in neutral phosphate solutions by the oxygen of the air. This oxidation, which is catalyzed by traces of metal, is dependent on the concentration of phosphate or, in the same way, of arsenate (29). It is especially rapid with trioses and with

triose phosphates (2). The rate of oxidation increases as the pH is shifted from 6.5 to 8.5. Exactly the same is true for the oxidation of glyceraldehyde phosphate by cozymase in the presence of phosphate or arsenate.

Such a conception of an addition product, which does not possess a second asymmetric carbon atom in the aldehyde group, but behaves towards oxidizing agents like a compound, seems to receive further support from the recent interesting model studies of Baer (30) on oxidative cleavage of α-keto acids by means of lead tetraacetate in the presence of alcohols and other substances possessing hydroxyl groups, including phosphoric acid. Here the initial formation of a loose physical or chemical addition product (perhaps an "association of molecules held together by weak physical forces") between the keto group and the alcoholic group is indispensable for the oxidative splitting. In this way acetylphosphoric acid can be formed from pyruvic and phosphoric acids (31) as in the biosynthesis of this substance, discovered by Lipmann (32).

If such a concept applies to our system, the chemical combination of phosphate with the glyceraldehyde monophosphate would take place only as a result of the oxidation of the loose aldehyde phosphate addition product. It was assumed in our former publications (14), preceding the discovery of the diphosphoglyceric acid of Negelein, that the phosphate uptake in this system was brought about by cozymase, which would act simultaneously as a phosphorylating and oxidizing coenzyme. The glyceraldehyde-3-phosphate reacting with cozymase in the presence of phosphate was called the "reaction form of sugar." We acknowledge fully the great progress achieved by Warburg and his coworkers in isolating the oxidizing enzyme and its reaction product, the diphosphoglyceric acid, whereby an intermediary step of the "coupling reaction" between phosphorylation and oxidation was demonstrated. Nevertheless, their statement (12), that our ideas had proved to be wrong in view of their alleged discovery of a diphosphoglyceraldehyde as the reaction form of sugar instead of a monophosphoglyceraldehyde, supposed by us, may perhaps turn out to be not only unjust but premature.

SUMMARY

Methods are described for determining very small amounts of *d*-3-glyceraldehyde phosphate in the presence of large amounts of dihydroxyacetone phosphate and hexose diphosphate. In this way the equilibrium constant of isomerase [dihydroxyacetone phosphate]/[glyceraldehyde phosphate] is found to be 20 to 25. The same value of 4 to 5 per cent glyceraldehyde phosphate in the total triose phosphate is obtained with preparations of zymohexase (aldolase + isomerase) and of purified isomerase.

The values for the equilibrium constant of aldolase,

$$\frac{[\text{Dihydroxyacetone phosphate}]\cdot[\text{glyceraldehyde phosphate}]}{[\text{Hexose diphosphate}]}$$

as determined with zymohexase or with purified aldolase, are also in complete agreement.

None of the equilibria is influenced by the presence of inorganic phosphate (0.04 to 0.1 M) even if the oxidizing enzyme of Warburg with or without cozymase is also added. No substance is formed which breaks down into glyceraldehyde phosphate and phosphate, as has to be assumed for a diphosphoglyceraldehyde.

The rôle of phosphate in the oxidation of glyceraldehyde phosphate by cozymase has to be sought, therefore, in the establishment of a loose physical addition product of these components, which would enable the formation of diphosphoglyceric acid by oxidation, without being in itself a definite chemical compound.

Acknowledgment is due to Miss Mildred Hurst for valuable help.

BIBLIOGRAPHY

1. Meyerhof, O., Biological symposia, Lancaster, **5**, 141 (1942).
2. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934).
3. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **279**, 40 (1935).
4. Meyerhof, O., *Biochem. Z.*, **277**, 77 (1935).
5. Meyerhof, O., Lohmann, K., and Schuster, P., *Biochem. Z.*, **286**, 301, 319 (1936).
6. Meyerhof, O., *Bull. Soc. chim. biol.*, **20**, 1033, 1345 (1938).
7. Meyerhof, O., Kiessling, W., and Schulz, W., *Biochem. Z.*, **292**, 25 (1937).
8. Negelein, E., and Brömel, H., *Biochem. Z.*, **301**, 135 (1939); **303**, 132, (1939).
9. Meyerhof, O., *Bull. Soc. chim. biol.*, **21**, 965 (1939).
10. Herbert, H., Gordon, V., Sabrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108 (1940).
11. Warburg, O., and Christian, W., *Biochem. Z.*, **301**, 201 (1939).
12. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
13. Kiessling, W., *Biochem. Z.*, **298**, 421 (1938).
14. Meyerhof, O., Ohlmeyer, P., and Mohle, W., *Biochem. Z.*, **297**, 113 (1938).
15. Fischer, H. O. L., and Baer, E., *Ber. chem. Ges.*, **65**, 337 (1932).
16. Meyerhof, O., and Schulz, W., *Biochem. Z.*, **297**, 60 (1938).
17. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
18. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443 (1942).
19. Schlenk, F., in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, Leipzig, 2297 (1940).
20. Jandorf, B. T., *J. Biol. Chem.*, **138**, 304 (1941).
21. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
22. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).
23. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **267**, 313 (1933).
24. Iri, S., *J. Biochem.*, Japan, **27**, 7 (1938).

25. Cori, C. F., Colowick, S. P., and Cori, G. T., *J. Biol. Chem.*, **121**, 465 (1937).
26. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **143**, 563 (1942).
27. Warburg, O., and Yabusoe, M., *Biochem. Z.*, **146**, 380 (1924).
28. Wind, F., *Biochem. Z.*, **159**, 58 (1925).
29. Meyerhof, O., and Matsuoka, K., *Biochem. Z.*, **150**, 1 (1924).
30. Baer, E., *J. Am. Chem. Soc.*, **62**, 1597 (1940); **64**, 1416 (1942).
31. Baer, E., *J. Biol. Chem.*, **146**, 391 (1942).
32. Lipmann, F., *J. Biol. Chem.*, **134**, 463 (1940).

ON THE COAGULATION OF FIBRINOGEN*

By ERWIN CHARGAFF and AARON BENDICH

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 26, 1943)

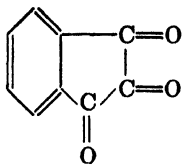
The nature of the chemical reactions that, in the presence of thrombin, lead to the formation of fibrin, the insoluble coagulation product of fibrinogen, is largely unknown. Because of the lack of reliable comparative analyses of the composition of pure fibrinogen and fibrin and of the amino acid distribution in these proteins, it is not even possible to decide whether the products obtained from fibrinogen by heat coagulation and by the action of thrombin are different. To speak, as is sometimes done, of thrombin as a "denaturase" (1, 2) is, at the present state of our knowledge, an addition to our terminology rather than to the understanding of the reactions involved.

Papain has been shown to coagulate fibrinogen directly (3), but this in itself is not sufficient indication of the proteolytic nature of the physiological coagulation phenomenon. It is, however, conceivable that with certain enzymes the formation of fibrin represents the first step in the proteolytic degradation of fibrinogen and that this initial phase is particularly protracted with thrombin as clotting agent. The literature contains reports on the fibrinolytic action of thrombin (4, 5), and experiments on the formation and lysis of fibrin by staphylococci may also be cited (6). For the time being, it would appear advisable to limit the definition of the action of thrombin to the statement that it brings about changes in the structure of fibrinogen that lead to the formation of the insoluble protein fibrin,[1] and to defer speculation on the nature of these changes to a time when more experimental data are available.

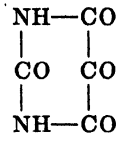
It has recently been found and briefly reported from this laboratory (7) that *ninhydrin* (1,2,3-indantrione hydrate) (I) coagulated fibrinogen and formed clots which in some respects closely resembled fibrin. *Alloxan* (II) and *salicylaldehyde* were likewise, although much less markedly, active. This observation led to a search for other synthetic agents having a

* This work has been supported by a grant from the John and Mary R. Markle Foundation. This is Paper XVI of a series of studies on the chemistry of blood coagulation.

[1] It will avoid misunderstandings if the term fibrin is reserved for the coagulation product obtained with *thrombin*. Insoluble products formed from fibrinogen with other clotting agents will be referred to as coagulated fibrinogen.

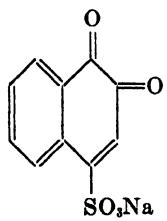


(I)

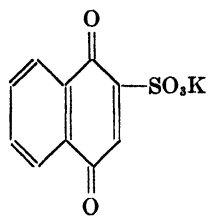


(II)

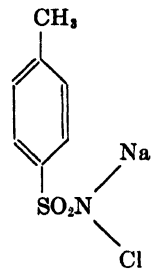
similar action. The sodium salt of *1,2-naphthoquinone-4-sulfonic acid* (III) and the potassium salt of *1,4-naphthoquinone-2-sulfonic acid* (IV) have been found to produce firm clots from solutions of purified fibrinogen at 30° and pH 7. *Chloramine-T* (sodium *p*-toluenesulfonchloroamide) (V), while acting even more rapidly under similar conditions, forms a loose coagulum.



(III)



(IV)



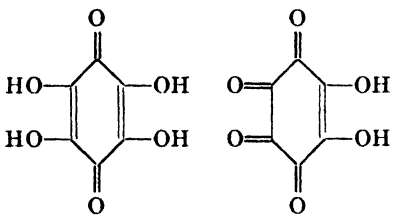
(V)

Neither the thromboplastic factor nor calcium was required in this reaction which proceeded with fibrinogen preparations free of prothrombin. The clotting times observed depended on the concentrations of both the fibrinogen and the clotting agent. In fibrinogen solutions of the strength used in the present investigation, the clotting agents, with the exception of chloramine-T, produced firm coherent clots. The retraction of the clots appeared to proceed at a faster rate than with fibrin produced by thrombin: a solid opaque core of protein began to form underneath the transparent gel a short time after the coagulation by the clotting agent in high concentration.

In a number of experiments, the recovery of nitrogen in the coagulation products was studied (Tables III and IV). With ninhydrin and the α- and β-naphthoquinone sulfonates, the amounts of fibrinogen N recovered in the clots were of the same order of magnitude as found in the corresponding fibrin samples. Alloxan, and perhaps also chloramine-T, appeared to attack the protein more energetically. The low sulfur content of the coagulation product obtained with alloxan (Table III, Experiment 4) may be significant. Compound formation between ninhydrin and fibrinogen

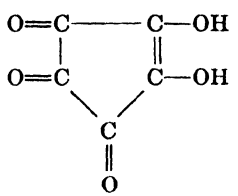
seemed to have occurred to only a slight extent, if at all. The somewhat higher sulfur contents of the fibrinogen samples clotted by the naphthoquinone sulfonates may indicate the presence of these agents in the clots. It is, however, so difficult to wash these coagulation products satisfactorily that mechanical adsorption cannot be excluded. It would undoubtedly be of interest to study the x-ray diffraction patterns of the coagulation products of fibrinogen.

The clotting activity of the ketones and quinones mentioned in the preceding paragraphs made it appear of interest to study a number of biologically occurring compounds that could, under physiological conditions, conceivably give rise to active substances. One of the systems studied was *mesoinositol* and the series of oxidation products derived from it; *viz.*, tetrahydroxyquinone (VI), rhodizonic acid (VII), croconic acid (VIII),[2] leuconic acid (IX). None of these compounds, however, exhibited any

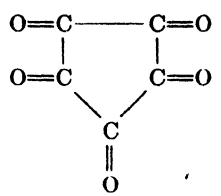


(VI)

(VII)



(VIII)



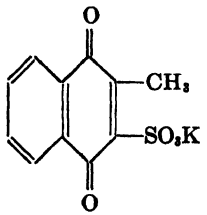
(IX)

clotting activity. Solutions containing *l-ascorbic acid* oxidized with iodine (8–10) or by the catalytic action of the ascorbic oxidase from squash (11) likewise had no effect on fibrinogen.

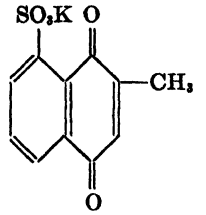
Another biological system studied, which could be considered most immediately related to the subject of this communication, was that of compounds belonging to the *vitamin K group*, of which 2-methyl-1,4-naphthoquinone is the most active representative (compare (12–14)). The examination of the possible direct rôle in clotting of compounds displaying vitamin K activity is, unfortunately, difficult. The quinones having vitamin K activity are not sufficiently soluble in water for clotting tests and all known water-soluble vitamin K analogues are derivatives of either the related hydroquinone or of naphthalene. Two water-soluble derivatives of 2-methyl-1,4-naphthoquinone were examined; *viz.*, the potassium salts of *2-methyl-1,4-naphthoquinone-3-sulfonic acid* (X) (15) and the new compound[3] *2-methyl-1,4-naphthoquinone-8-sulfonic acid* (XI). The antihemorrhagic activity of compound (X) is only slight ((15), compare also (16)) and that of compound (XI) has not yet been established, but it is

[2] The position of the hydroxyls in compounds (VII) and (VIII) is not quite certain.

[3] Chargaff, E., and Bendich, A., unpublished results.



(X)



(XI)

known that the introduction of a methyl group into position 8, as in 2,8-dimethyl-1,4-naphthoquinone (17), destroys the antihemorrhagic activity almost completely (13). Compound (X) proved inactive as a clotting agent. As this was possibly due to the absence of a free 3 position, compound (XI) was prepared, but it, too, failed to coagulate fibrinogen.

It thus cannot be proposed at present that vitamin K, or a compound to which it gives rise in the body, is directly involved in the coagulation of fibrinogen; *e.g.*, as a prosthetic group in the prothrombin or thrombin molecule. The contrary may, in fact, be true; *viz.*, that quinones displaying vitamin K activity are structurally protected from entering into reactions with the body proteins (of which the clotting of fibrinogen may be only one instance) that would prevent them from reaching the liver. A similar suggestion was first put forward by Fieser and collaborators ((13) p. 668).

A list of other carbonyl compounds which were tested for fibrinogen clotting and found inactive will be given in the experimental part. It is, perhaps, noteworthy that the triketone *2,3,4-triketopentane*, which may be considered the simplest aliphatic analogue of ninhydrin, failed to coagulate fibrinogen.

The discussion of the possible mechanism of action of the synthetic clotting agents on fibrinogen will best start from the fact that most of the compounds found to be active are able to oxidize amino acids and peptides containing free amino groups. This effect has been shown for chloramine-T (18), 1,2-naphthoquinone-4-sulfonic acid (19), ninhydrin (20, 21), and alloxan (22). For purposes of comparison the action on alanine of some of the clotting agents and also of several carbonyl compounds devoid of coagulant activity was studied. It will be seen from the data presented in this paper that, with one significant exception, the ability of the compounds to decarboxylate the amino acid paralleled their activity in the coagulation of fibrinogen. Generally, substances that failed to liberate CO_2 from alanine were inactive in clotting, with the exception of 1,4-naphthoquinone-2-sulfonic acid which, although an active clotting agent, did not decarboxylate alanine.

The very pronounced reactivity of 1,2-naphthoquinone and its 4-sulfonic

acid is well known and has been particularly stressed in the procedures developed by Ehrlich and Herter (23) and by Folin (24). Amines are added in position 4 (25, 26), in the case of the 4-sulfonate with the simultaneous liberation of bisulfite (27). It is, however, not quite clear whether the mechanism of the reaction with amino acids and, in particular, of the concomitant liberation of CO_2 is similar to that formulated for ninhydrin (20, 21). Compare also the observations on the reaction between proteins and ninhydrin (28) and 1,2-naphthoquinone-4-sulfonic acid (29). 1,4-Naphthoquinone is known to condense in position 2 with *p*-aminobenzoic acid (30) and other amines (31, 32). In the absence of direct information on the reaction of α-naphthoquinone with amino acids an analogy may be cited; *viz.*, the behavior of *p*-quinone towards glycine esters, studied by Fischer and Schrader (33), where addition in positions 2 and 5 of the quinone was observed. The reactions between 1,4-naphthoquinone-2-sulfonic acid and amines have not been sufficiently investigated (34).

There would appear to be several possible mechanisms of the clotting of fibrinogen by the synthetic agents; *e.g.*, (1) oxidation of aminoacyl groups present in the protein, (2) oxidation of other susceptible groupings, such as sulfhydryl (35), (3) combination of the clotting agents with the protein, perhaps through the phenolic group of tyrosine, as in the complex compounds between 1,4-naphthoquinone and various phenols (36). The systems considered in this study are too complicated to permit a consideration of the possible rôle of semiquinones (37) in the coagulation of fibrinogen by quinones.

Mechanism (1) agrees best with the experimental analogies reviewed before. If the oxidation proceeded by way of an amino acid to a keto acid, the breakdown of the latter to give CO_2 could in certain circumstances be expected. The conditions, however, under which clotting experiments have to be performed (low temperature, short duration of the experiment) are not such as would favor the decarboxylation of a keto acid. Experiments in which large amounts of fibrinogen were rapidly clotted by ninhydrin failed to reveal, by a sensitive micro conductometric method, the liberation of CO_2.

Reducing substances, as sodium bisulfite and glutathione, were found to inhibit the action of the clotting agents. The effect of glutathione on 1,2-naphthoquinone-4-sulfonic acid was not very marked. Sodium arsenite and sodium isoascorbate were ineffective as inhibitors.

When the conception of the clotting of fibrinogen as an oxidative process is extended to the formation of fibrin by thrombin, it will be understood why the chemical differences between fibrinogen and fibrin are, at least from what little we know about the composition of these proteins, so small. The removal from the large molecule of a few polar groups or the blocking

of these groups would not produce a noticeable change in the analytical figures. But a reaction of this type (whether it is to be called proteolytic is a matter of definition) might be sufficient to bring about a conspicuous and irreversible change in the solubility of a large molecule.

Attempts were made to analyze the composition of the atmosphere above a clotting mixture both before and after the addition of thrombin to fibrinogen. Thanks to the collaboration of Dr. D. Rittenberg, the CO_2 concentrations were measured in the mass spectrometer. In several experiments with large amounts of purified fibrinogen, indications of the liberation during clotting of very small amounts of CO_2 were obtained, but the results were not always reproducible. It may be mentioned that in these experiments rapid coagulation of fibrinogen by thrombin was observed in an atmosphere of water vapor at a nitrogen pressure of 10^{-4} mm. of Hg. The nitrogen contained less than 0.1 per cent of oxygen. This obviously indicates that the action of thrombin requires no atmospheric oxygen.

Experiments on the effect of various reducing agents on the coagulation of freshly prepared fibrinogen by thrombin demonstrated the inhibiting action of sodium bisulfite, which is in line with similar results obtained with the synthetic clotting agents. Other reducing substances were without effect. It will be remembered that under different experimental conditions cysteine (38), which was not used in the present study, and glutathione (39) have been found inhibiting. It should be borne in mind that it is at present not possible to decide whether the reducing agent acts on the thrombin or on the fibrinogen, but by analogy to the behavior of the synthetic clotting agents, the first assumption appears preferable.

We hope to continue these studies with an investigation of the behavior of other oxidizing agents and other quinones and of additional biological systems; *e.g.*, the flavins and the oxidation products of adrenalin.

EXPERIMENTAL

Fibrinogen

Preparation—Most experiments were carried out with fibrinogen preparations that were freshly obtained from human or horse plasma.[4] In a typical experiment, 25 gm. of washed $Ca_3(PO_4)_2$ were added to 600 cc. of citrated horse plasma, in order to remove prothrombin (40). The mixture was centrifuged 10 minutes later in an angle centrifuge at 4700 R.P.M. The precipitate produced by the addition to the supernatant of 900 cc. of saturated sodium chloride solution was removed by centrifugation, dis-

[4] We should like to express our appreciation for the courtesy of Dr. W. G. Malcolm, the Lederle Laboratories, Inc., Pearl River, New York, who supplied large amounts of horse plasma. We are also indebted for some horse plasma to E. R. Squibb and Sons, New Brunswick, New Jersey.

solved in 160 cc. of 0.14 M NaCl solution, and reprecipitated by the addition of 200 cc. of saturated NaCl solution. After one or two additional precipitations from its solution in 100 cc. of 0.14 M NaCl by means of an equal volume of saturated NaCl solution, the protein was dissolved in 0.14 M NaCl and the centrifuged solution used in the experiments. All operations were carried out at a low temperature with ice-cold fluids containing a trace of potassium oxalate. The fibrinogen solutions usually employed had a concentration of between 0.4 and 0.7 per cent.[5] All experiments were performed within a few days after the preparation of the protein, during which period the solution was stored in the frozen state at −40°.

Coagulation—Unless otherwise indicated, the clotting experiments were carried out at pH 6.8 to 7.0 in a water thermostat kept at 30.5°, with small Pyrex tubes (10 × 75 mm.) which contained 0.2 cc. of the fibrinogen and 0.03 cc. of the test or control solutions.

Clotting Effect of Ninhydrin

It was shown in a previous communication from this laboratory (7) that *ninhydrin* (1,2,3-indantrione hydrate) produced clots when added to a solution of fibrinogen. Eastman Kodak preparations of ninhydrin were used in all experiments. This reaction did not require prothrombin, calcium, or the thromboplastic factor. In the formation of fibrinogen clots by ninhydrin (*cf.* (7)), a slight blue tint appeared when the clotted mixture was heated or stored for more than 2 hours.

The clotting time depended on the concentrations of both the fibrinogen and the ninhydrin, as shown in Table I. If the fibrinogen concentration was kept constant, smooth curves were obtained by plotting the clotting time against ninhydrin concentration. These curves were fairly well reproducible for different fibrinogen preparations. It was, consequently, possible by extrapolation to estimate the fibrinogen concentration of a solution by determining the time required for its coagulation by a known amount of ninhydrin.

Clotting Effect of Quinones

Preparation of Material—The *potassium salt of 1,4-naphthoquinone-2-sulfonic acid* was prepared from 1,4-naphthoquinone according to the method of Fieser and Fieser (41).

$C_{10}H_5O_5SK$ (276.3). Calculated, S 11.60, K 14.16; found, S 11.40, K 14.00

The *sodium salt of 1,2-naphthoquinone-4-sulfonic acid* was an Eastman Kodak preparation, purified by recrystallization.

[5] The results obtained with too dilute fibrinogen solutions are erratic and unreliable. In all experiments reported here the amounts of fibrinogen N used, a more exact measure than the percentage of fibrinogen, are indicated.

The *potassium salt of 2-methyl-1,4-naphthoquinone-3-sulfonic acid* was prepared from 2-methyl-1,4-naphthoquinone[6] according to Moore (15).

$C_{11}H_7O_5SK$ (290.3). Calculated, S 11.05, K 13.48; found, S 11.25, K 13.49

The synthesis of the *potassium salt* of *2-methyl-1,4-naphthoquinone-8-sulfonic acid* will be reported in a separate publication. This new compound was obtained by the oxidation of 2-methylnaphthalene-8-sulfonamide to 2-methyl-1,4-naphthoquinone-8-sulfonamide.

Clotting Action—The coagulation of human fibrinogen by *potassium 1,4-naphthoquinone-2-sulfonate* and the influence of the relative concentrations of the two reactants on the coagulation time are demonstrated in Table I.

TABLE I

Coagulation of Fibrinogen by Ninhydrin and Potassium 1,4-Naphthoquinone-2-sulfonate

Columns *a* represent clotting by ninhydrin (1 micromole = 178.1 γ); Columns *b*, clotting by potassium α-naphthoquinone sulfonate (1 micromole = 267.3 γ). Each tube contained 0.2 cc. of the solution of human fibrinogen and 0.03 cc. of the solution of the clotting agent. The experiments were carried out at 30.5° and pH 6.8.

Fibrinogen N in experiment	Clotting agent in experiment, micromoles								
	3.5		1.75		0.88		0.44		0
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	
γ	*min.*	*min.*	*min.*	*min.*	*min.*	*min*	*min.*	*min.*	*min*
620	5	3	9	4	17	5	35	9	>240
310			14	6					
155			18	8					
78			21	13					

The clotting effect of *sodium 1,2-naphthoquinone-4-sulfonate* is shown in Table II, with horse fibrinogen as substrate. The coagulation of the same protein by the 1,4-naphthoquinone-2-sulfonate is likewise included. In general, the most rapid firm coagulation of fibrinogen was observed with the 1,4-naphthoquinone sulfonate, followed in this order, by the 1,2-naphthoquinone sulfonate and ninhydrin.

The *potassium salts* of *2-methyl-1,4-naphthoquinone-3-sulfonic acid* and of *2-methyl-1,4-naphthoquinone-8-sulfonic acid* exhibited no clotting activity.

[6] We wish to thank Dr. John Lee of Hoffmann-La Roche, Inc., Nutley, New Jersey, for the starting material.

Additional Clotting Agents

Only a few of the numerous compounds tested were found to coagulate fibrinogen. They were *chloramine-T* (sodium *p*-toluenesulfonchloroamide), *alloxan*, and *salicylaldehyde*. Chloramine-T produced more rapid clotting than any other synthetic agent, but the resulting coagulum was extremely soft and loose. The following coagulation experiment was carried out at pH 7 with a preparation of human fibrinogen (650 γ of fibrinogen N in 0.2 cc.) and solutions of neutralized chloramine-T.

Chloramine-T in experiment, *micromoles* ..	5	2.5	1.25	0.63	0
Clotting time, *min.* ..	<1	1	4	7	>120

TABLE II

Coagulation of Fibrinogen by Quinones

Each tube contained 0.2 cc. of the solution of horse fibrinogen, corresponding to 125 γ of fibrinogen N, and 0.03 cc. of the solution of the clotting agent. The experiments were carried out at 30.5° and pH 6.8.

	Clotting agent in experiment, micromoles					
	3.5	1.75	0.88	0.44	0.22	0
	min.	*min.*	*min.*	*min.*	*min.*	*min.*
Potassium 1,4-naphthoquinone-2-sulfonate (1 micromole = 267.3 γ)	6	8	10	25	>120	>120
Sodium 1,2-naphthoquinone-4-sulfonate (1 micromole = 260.2 γ)	11	15	25	47	>120	>120

The effects of the two other agents mentioned were extremely weak. Alloxan coagulated 0.2 cc. of an 0.8 per cent solution of human fibrinogen in 28 minutes in a concentration of 10 micromoles and in 60 minutes in a concentration of 5 micromoles. The addition of 0.03 cc. of a saturated solution of salicylaldehyde in physiological saline to 0.2 cc. of the same fibrinogen preparation produced clotting in 40 minutes.

Analysis of Coagulated Fibrinogen Samples

In one series of experiments, the nitrogen distribution following coagulation and the composition of the clotted fibrinogen samples resulting from the coagulation of purified *horse fibrinogen* by *thrombin*, *ninhydrin*, and *alloxan* were followed quantitatively. The thrombin preparation used was obtained from human plasma by the method of Eagle (42). The fibrinogen solutions and the solutions of the clotting agents (in physiological saline) were adjusted to neutrality. Following the addition of the

clotting agents, the mixtures were kept at room temperature for ½ hour and subsequently stored in the refrigerator for 48 hours. They were then centrifuged, and the precipitates washed with four 10 cc. portions of ice-cold water and dried *in vacuo* over P_2O_5. The united supernatants and washings were adjusted to an exact volume of 100 cc. and aliquots analyzed for nitrogen. In the experiment with thrombin, allowance was made for the amount of nitrogen introduced as thrombin. The dried samples of coagulated protein were, for analysis, allowed to equilibrate with air and their moisture and ash contents determined in separate analyses, in order to arrive at the corrected analytical figures reproduced in Table III. The coagulation product obtained by ninhydrin was steel-blue before and slightly yellow after washing.

In another set of experiments the coagulation of *human fibrinogen* by *beef thrombin*,[7] *ninhydrin*, *1,4-naphthoquinone-2-sulfonate*, *1,2-naphtho-*

TABLE III

Quantitative Analysis of Coagulated Horse Fibrinogen Preparations

Experiment No.	Clotting agent	Volume of reaction mixture	Fibrinogen N used	Coagulum N recovered		N in supernatant	Analyses of coagulated fibrinogen	
							N	S
		cc.	*mg.*	*mg.*	*per cent of fibrinogen N used*	*mg.*	*per cent*	*per cent*
1	Thrombin	20.5	18.3	16.0	87.4	2.9	15.4	0.98
2	Ninhydrin, 54 mg.	22	21.6	18.6	86.1	3.0	14.6	1.07
3	" 177.6 "	35	22 8	19 1	83.8	3.4	15.0	1.02
4	Alloxan, 213.9 "	30	22.8	8 2	36.0		13.1	0 56

quinone-4-sulfonate, and *chloramine-T* was examined. The clotting agent, dissolved in 2 cc. of M/15 phosphate buffer of pH 6.83, was added with constant stirring to 10 cc. of the buffered fibrinogen solution. After 2 hours at 26° the clots were broken up, centrifuged off, and washed twice with 3 cc. portions of physiological saline and three times with the same amounts of distilled water. The analytical procedure was similar to that described in the preceding paragraph. The results, corrected as above, are summarized in Table IV. It may be mentioned that in this experiment the clots, with the exception of the white fibrin produced by thrombin, exhibited the following colors in both the wet and the dry states: violet with ninhydrin and β-naphthoquinone sulfonate, deep orange-red with α-naphthoquinone sulfonate, light pink with chloramine-T.

[7] We are highly indebted for this preparation to Dr. D. W. MacCorquodale of the Abbott Laboratories, North Chicago, Illinois.

Influence of Reducing Substances on Fibrinogen Coagulation

The effect of reducing substances on the coagulation of fibrinogen by synthetic clotting agents and by thrombin was investigated with sodium bisulfite ($NaHSO_3$), glutathione, sodium arsenite ($NaAsO_2$), and sodium *d*-araboascorbate.[8] The action of these compounds on the clotting of

TABLE IV

Quantitative Analysis of Coagulated Human Fibrinogen Preparations

Experiment No.	Clotting agent		Coagulum N recovered (30.3 mg fibrinogen N used)		N in supernatant	Analyses of coagulated fibrinogen	
						N	S
		mg.	*mg.*	*per cent of fibrinogen N used*	*mg.*	*per cent*	*per cent*
1	Thrombin	4.0	28.0	92.4	2.3	17.6	1.12
2	Ninhydrin	15 0	26.9	88 8	2 4	16 1	1.21
3	Potassium 1,4-naphthoquinone-2-sulfonate	23 2	26 1	86.1	3 5	16 3	1.69
4	Sodium 1,2-naphthoquinone-4-sulfonate	22 0	27 3	90 1	1 9	16.3	1.41
5	Chloramine-T	23 8	20 6	68 0	8 7	16 7	1 34

TABLE V

Effect of Sodium Bisulfite on Coagulation of Human Fibrinogen by Quinones

Each tube contained 0.03 cc. of the solution of the clotting agent and 0.03 cc. of the $NaHSO_3$ solution dissolved in M/15 phosphate buffer of pH 6.8. 2 minutes later 0.2 cc. of a neutral solution of human fibrinogen (corresponding to 0.10 mg. of fibrinogen N) was added. The experiments were carried out at 30.5°.

Sodium bisulfite in experiment	Clotting agent in experiment, micromoles				Control
	Potassium 1,4-naphthoquinone-2-sulfonate		Sodium 1,2-naphthoquinone-4-sulfonate		
	3.5	1.75	3.5	1.75	
micromoles	*min*	*min.*	*min*	*min.*	*min.*
3.5	20	60	>90	>90	>90
0	5	8	11	17	>90

fibrinogen (from human and horse plasma) by organic clotting agents is shown in Tables V and VI. It will be seen that sodium bisulfite and glutathione markedly inhibited the action of the synthetic clotting agents. In order to establish whether the inhibiting action of glutathione was due to

[8] We should like to thank Dr. H. M. Wuest of Hoffmann-La Roche, Inc., Nutley, New Jersey, for this compound.

its reducing properties or to its free —$CH(NH_2)\cdot COOH$ group by means of which it could react with the clotting agents, parallel experiments were carried out in which alanine was substituted for glutathione. No clotting inhibition was observed. The substitution of sodium sulfate for sodium bisulfite likewise proved ineffective.

TABLE VI

Effect of Reducing Substances on Coagulation of Horse Fibrinogen by Synthetic Clotting Agents

Each tube contained 0.1 cc. of the solution of the clotting agent and 0.1 cc. of that of the reducing agent dissolved in M/15 phosphate buffer of pH 6.9. 2 minutes later 0.2 cc. of horse fibrinogen solution (corresponding to 0.2 mg. of fibrinogen N) was added. The experiments were carried out at 30.5°.

Reducing substance in experiment		Clotting agent (12 micromoles in experiment)		
		Ninhydrin	Potassium 1,4-naphthoquinone-2-sulfonate	Sodium 1,2-naphthoquinone-4-sulfonate
	micromoles	*min.*	*min.*	*min.*
Glutathione	12	40	>80	8
	6	28	17	6
Sodium arsenite	12	9	5	
	6	9	5	
" *d*-araboascorbate	12	11	5	
	6	11	5	
Control		9	5	4

TABLE VII

Effect of Sodium Bisulfite on Coagulation of Horse Fibrinogen by Thrombin

Each tube contained 0.05 mg. of beef thrombin dissolved in 0.1 cc. of M/15 phosphate buffer of pH 6.8 and 0.1 cc. of the $NaHSO_3$ solution in the same buffer. 2 minutes later 0.2 cc. of the neutral fibrinogen solution (corresponding to 0.37 mg. of fibrinogen N) was added. The experiments were carried out at 30.5°.

	Sodium bisulfite in experiment, micromoles				
	12	6	3	1.5	0
Clotting time, *min.*.. ..	58	8	4	2	1

The effect of the reducing agents mentioned above on the coagulation of horse fibrinogen by beef thrombin (obtained from the Abbott Laboratories) was likewise examined. In these experiments only sodium bisulfite proved effective (Table VII).[9] With a dilute solution of human fibrinogen (0.10

[9] The somewhat different behavior of dry fibrinogen preparations will be discussed at a later date.

mg. of fibrinogen N in the experiment) the following clotting times were observed at room temperature (27°), when constant amounts of thrombin and decreasing amounts of $NaHSO_3$ were employed: with 13.5 micromoles of $NaHSO_3$, more than 20 minutes; with 1.35 micromoles, 182 seconds; with 0.135 micromole, 124 seconds; in absence of $NaHSO_3$, 107 seconds.

Examination of Atmosphere above Clotting Mixture in Mass Spectrometer

It appeared of interest to investigate the possibility of demonstrating the liberation of very small amounts of carbon dioxide during the coagulation of fibrinogen by thrombin. Thanks to the generous collaboration of Dr. D. Rittenberg, a number of experiments could be carried out in which the CO_2 concentration in the atmosphere above a fibrinogen solution both before and after the addition of thrombin was determined by means of the mass spectrometer (43).

The fibrinogen solutions used were freshly prepared from horse plasma and contained a total of between 15 and 40 mg. of fibrinogen N in a volume of 30 cc. of physiological saline. The thrombin solutions contained 10 to 20 mg. of beef thrombin (obtained from the Abbott Laboratories) in a volume of 5 cc. of saline. The fibrinogen solution was adjusted to pH 5.8, the thrombin solution to pH 5.9. Nitrogen, free of CO_2, was passed through both solutions for about 10 minutes, following which they were carefully degassed by evacuation with a Hyvac pump. CO_2-free air was admitted and the pH of the solutions again determined and, when necessary, readjusted to the values given above.

The fibrinogen solution, in a 250 cc. flask carrying by means of a side arm a smaller bulb containing the thrombin solution, was then connected with the mass spectrometer line, and the system repeatedly flushed with nitrogen and evacuated, until the detectable amount of mass 44 (CO_2) was minimal. At this point the thrombin solution was tipped in, whereupon clotting invariably took place in a very short time. (In a few experiments this operation was preceded by the addition of heat-inactivated thrombin from a second side bulb.) It is noteworthy that under the conditions of the experiments rapid coagulation of fibrinogen was observed in a nitrogen atmosphere (containing less than 0.1 per cent of oxygen) at a permanent gas pressure of about 10^{-4} mm. of Hg.; *i.e.*, with a partial oxygen pressure of about 10^{-7} mm. of Hg. At each step and also at various intervals after clotting, samples of the atmosphere above the clotting mixture were removed in which the ratio of mass 44 to mass 29 ($N^{14}N^{15}$) was determined. In several experiments indications of the liberation during clotting of small amounts of CO_2 were obtained, but the results were not sufficiently reproducible to warrant their detailed presentation at this point. It may be mentioned that the pH of the clotted mixtures, determined at the con-

clusion of the experiments, was always found to have remained almost unchanged.

Attempts were also made to detect the liberation of CO_2 during clotting by means of the very sensitive micro conductometric arrangement described by Witte (44). With the circulating absorption conductivity cell used in these experiments it was possible to determine as little as 5 micromoles of CO_2 (220 γ) with satisfactory accuracy. With fibrinogen solutions, similar to the preparations described at the beginning of this section, no liberation of CO_2 could be demonstrated when thrombin or ninhydrin was used as clotting agent. In these experiments, too, we were aided by the collaboration and the advice of Dr. D. Rittenberg.

Clotting Agents and Amino Acid Oxidation

The ability of the various clotting agents and also of some of the inactive carbonyl compounds to liberate CO_2 from amino acids was studied with *dl*-alanine as substrate. With carbonyl compounds that were not volatile with water vapor, the microtitration procedure of Van Slyke and collaborators (45) was employed; the other substances were examined according to the manometric micromethod of Van Slyke *et al.* (19) in the Van Slyke-Neill apparatus. In the latter procedure, especially when extended reaction times were desired, small glass tubes with ground-in glass stoppers were found valuable for the reaction between the carbonyl compound and the amino acid. The experimental results are presented in Table VIII. The values are corrected for the amounts of CO_2 spontaneously given off by the compounds examined.

Inactive Compounds

Carbonyl Compounds—Various carbonyl compounds were tested for their action on fibrinogen and found inactive in the concentrations employed in this study. Among them may be mentioned formaldehyde, acetone, pyruvic acid, diacetyl, dihydroxytartaric acid, 2-ketoglutaric acid, 2,3,4-triketopentane, and phloroglucinol.

2,3,4-Triketopentane was synthesized from *p*-nitrosodimethylaniline and acetylacetone according to Sachs and Röhmer (46). The yellow oil (b.p. 56–58° under 13 mm. of Hg) was characterized by its disemicarbazone, white crystals from acetic acid melting at 223° (46), and its dianil (47), crystals from ethyl alcohol melting at 155°.

Inositol and Its Oxidation Products—The entire series of products resulting from the oxidation of mesoinositol was examined for its action on fibrinogen. The compounds prepared and tested were (1) tetrahydroxyquinone, (2) rhodizonic acid, (3) croconic acid, (4) leuconic acid.

Dipotassium tetrahydroxyquinone and *dipotassium rhodizonate* were prepared from inositol according to the method of Preisler and Berger (48).

Croconic acid was prepared from rhodizonic acid (49). It is difficult to obtain this interesting compound in a satisfactory yield. The following method was found to give the best results. The oxidation of 20 gm. of inositol with concentrated nitric acid, followed by aeration for 19 hours of the oxidation product in potassium acetate solution (48), yielded 9.7 gm. of the beautiful purple prisms of dipotassium rhodizonate. The crystals were suspended in 150 cc. of water, 20 cc. of 2 N KOH were added, and the mixture was heated to boiling for 18 minutes. With small samples, re-

TABLE VIII

Reaction between Carbonyl Compounds and dl-Alanine

Experiment No.*	Carbonyl compound used		Method †	Boiling time for reaction	CO_2 liberated	
		mg.		*min.*	*micromoles*	*per cent of theory*
1	Ninhydrin	50	M.	6	44.2	99.4
2	"	20	T.	7	44.4	99.7
3	Alloxan‡	16	"	10	5.7	13
4	Sodium 1,2-naphthoquinone-4-sulfonate	30	"	7	32.6	73.2
5	Sodium 1,2-naphthoquinone-4-sulfonate	30	"	30	34.2	76.8
6	Potassium 1,4-naphthoquinone-2-sulfonate	30	"	7	0	0
7	Potassium 1,4-naphthoquinone-2-sulfonate	30	"	60	1.4	3
8	1,4-Naphthoquinone	28	M.	15	0.3	0
9	Potassium 2-methyl-1,4-naphthoquinone-3-sulfonate	30	T.	20	0	0
10	2-Methyl-1,4-naphthoquinone	30	M.	15	0	0
11	Dipotassium tetrahydroxyquinone‡	30	T.	15	1.9	4
12	Dipotassium rhodizonate‡	30	"	15	3.0	7
13	Dipotassium croconate	30	M.	15	0.3	0
14	Diacetyl	10	"	15	0.2	0
15	2,3,4-Triketopentane‡	13	"	15	1.9	4

* In each experiment 44.5 micromoles of *dl*-alanine were used. The reactions were carried out in citrate buffer of pH 4.7.

† M. designates the manometric (20), T. the titrimetric (45) procedure.

‡ With this compound considerable spontaneous evolution of CO_2 was observed.

moved at various intervals during this treatment, test precipitations of the barium salt were made. When the brick-red color of this salt had changed to a pure yellow, the solution was allowed to cool, and was filtered and concentrated *in vacuo* to a volume of 30 cc. The crude product, obtained as needles on brief cooling, was three times recrystallized from small amounts of water in the presence of norit, when the beautiful yellow needles of *dipotassium croconate*, weighing 1.3 gm., were obtained. For analysis, the compound was dried *in vacuo* at 110°.

$C_5O_5K_2$ (218.2). Calculated, K 35.8; found, K 36.0

The recovery of additional material from the united mother liquors was laborious and had to proceed through the barium salt. In this manner 0.4 gm. of potassium croconate was isolated. The total amounted to 20 per cent of the theoretical yield.

Leuconic acid was prepared in solution by the oxidation of potassium croconate with the equivalent amount of nitric acid (50, 51). When the solution of leuconic acid was adjusted to neutrality, to test for its clotting activity, great care had to be taken to avoid an alkaline reaction, since otherwise decomposition and precipitation of sodium mesoxalate (52) took place.

Inositol and croconic and leuconic acids were tested with fibrinogen in a maximum concentration of 3.5 micromoles in the experiment; tetrahydroxyquinone and rhodizonic acid had to be tested in lower concentrations (1 micromole) because of their scant solubility in water. All compounds were inactive.

Ascorbic Acid and Its Oxidation Products—Attempts were made to test the effect on fibrinogen of the oxidation products of ascorbic acid. The oxidation of solutions of ascorbic acid with the required amount of iodine followed by careful neutralization led to inactive solutions. Since dehydroascorbic acid is known to be unstable *in vitro* above pH 4, experiments were also carried out at pH 6.8 with an ascorbic acid oxidase preparation freshly obtained from summer yellow squash (11). The incubation of ascorbic acid and fibrinogen in presence of the ascorbic oxidase, however, did not produce coagulation.

The authors are very grateful to Mr. W. Saschek for a number of microanalyses.

SUMMARY

The following substances in low concentration were found to coagulate fibrinogen (in the order of their activity): chloramine-T, potassium 1,4-naphthoquinone-2-sulfonate, sodium 1,2-naphthoquinone-4-sulfonate, ninhydrin, and, much less markedly, alloxan and salicylaldehyde.

Some of the theoretical implications of the coagulation of fibrinogen by quinones were pointed out. Since most of the active clotting agents have been known to oxidize amino acids and certain peptides, the action of the thrombin on fibrinogen may likewise be of an oxidative nature.

Because of the importance of vitamin K in blood clotting, two sulfonic acid derivatives of 2-methyl-1,4-naphthoquinone were tested with fibrinogen, but proved inactive. Other biological systems examined, which also were ineffective, were ascorbic acid, inositol, and their oxidation products.

BIBLIOGRAPHY

1. Wöhlisch, E., *Klin. Woch.*, **2**, 1801 (1923).
2. Strughold, H., and Wöhlisch, E., *Z. physiol. Chem.*, **223**, 267 (1934).
3. Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, **20**, 543 (1937).
4. Hirose, R. S., *Am. J. Physiol.*, **107**, 693 (1934).
5. Jaques, L. B., *Biochem. J.*, **32**, 1181 (1938).
6. Fisher, A. M., *Bull. Johns Hopkins Hosp.*, **59**, 393, 415 (1936).
7. Chargaff, E., and Ziff, M., *J. Biol. Chem.*, **138**, 787 (1941).
8. Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., *J. Chem. Soc.*, 1270 (1933).
9. Haworth, W. N., and Hirst, E. L., in Mellanby, E., and Ruzicka, L., Ergebnisse der Vitamin- und Hormonforschung, Leipzig, **2**, 160 (1939).
10. Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, **117**, 237 (1937).
11. Lovett-Janison, P. L., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 1409 (1940).
12. Dam, H., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 285 (1942).
13. Fieser, L. F., Tishler, M., and Sampson, W. L., *J. Biol. Chem.*, **137**, 659 (1941).
14. Doisy, E. A., Binkley, S. B., and Thayer, S. A., *Chem. Rev.*, **28**, 477 (1941).
15. Moore, M. B., *J. Am. Chem. Soc.*, **63**, 2049 (1941).
16. Baker, B. R., Davies, T. H., McElroy, L., and Carlson, G. H., *J. Am. Chem. Soc.*, **64**, 1096 (1942).
17. Tishler, M., Fieser, L. F., and Wendler, N. L., *J. Am. Chem. Soc.*, **62**, 2866 (1940).
18. Dakin, H. D., *Biochem. J.*, **11**, 79 (1917).
19. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
20. Ruhemann, S., *J. Chem. Soc.*, **99**, 792 (1911).
21. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **509**, 288 (1934).
22. Strecker, A., *Ann. Chem.*, **123**, 363 (1862).
23. Ehrlich, P., and Herter, C. A., *Z. physiol. Chem.*, **41**, 379 (1904).
24. Folin, O., *J. Biol. Chem.*, **51**, 377 (1922).
25. Zincke, T., *Ber. chem. Ges.*, **14**, 1493 (1881).
26. Liebermann, C., and Jacobson, P., *Ann. Chem.*, **211**, 36 (1882).
27. Böniger, M., *Ber. chem. Ges.*, **27**, 23 (1894).
28. Dulière, W. L., *Biochem. J.*, **30**, 770 (1936).
29. Obo, F., *J. Biochem.*, Japan, **33**, 241 (1941).
30. Hauschka, R., *J. prakt. Chem.*, **90**, 449 (1914).
31. Zincke, T., *Ber. chem. Ges.*, **12**, 1641 (1879).
32. Grossmann, E., *J. prakt. Chem.*, **92**, 370 (1915).
33. Fischer, E., and Schrader, H., *Ber. chem. Ges.*, **43**, 525 (1910).
34. Seidel, P., *Ber. chem. Ges.*, **25**, 423 (1892).
35. Baumberger, J. P., *Am. J. Physiol.*, **133**, P206 (1941).
36. Pfeiffer, P., Organische Molekülverbindungen, Leipzig, 2nd edition, 293, 295 (1927).
37. Michaelis, L., *Ann. New York Acad. Sc.*, **40**, 39 (1940).
38. Mueller, J. H., and Sturgis, S., *Science*, **75**, 140 (1932).
39. Kühnau, J., and Morgenstern, V., *Z. physiol. Chem.*, **227**, 145 (1934).
40. Bordet, J., *Compt. rend. Soc. biol.*, **83**, 576 (1920).
41. Fieser, L. F., and Fieser, M., *J. Am. Chem. Soc.*, **57**, 491 (1935).
42. Eagle, H., *J. Gen. Physiol.*, **18**, 531 (1935).

43. Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, **127**, 291 (1939).
44. Witte, P. J., Dissertation, Columbia University (1933).
45. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).
46. Sachs, F., and Röhmer, A., *Ber. chem. Ges.*, **35**, 3307 (1902).
47. Sachs, F., and Barschall, H., *Ber. chem. Ges.*, **34**, 3047 (1901).
48. Preisler, P. W., and Berger, L., *J. Am. Chem. Soc.*, **64**, 67 (1942).
49. Nietzki, R., and Benckiser, T., *Ber. chem. Ges.*, **18**, 499, 1833 (1885).
50. Will, H., *Ann. Chem.*, **118**, 177 (1861).
51. Nietzki, R., and Benckiser, T., *Ber. chem. Ges.*, **19**, 293 (1886).
52. Homolka, B., *Ber. chem. Ges.*, **55**, 1310 (1922).

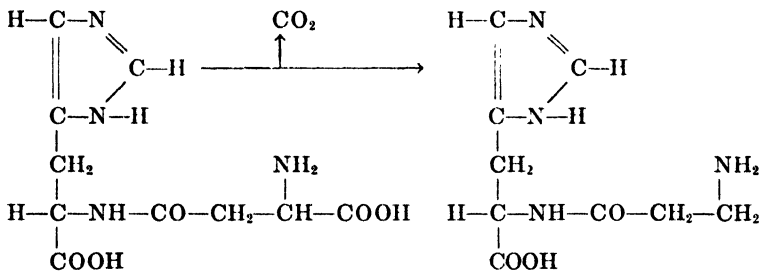
ON THE ORIGIN OF β-ALANINE IN THE ANIMAL BODY AND A MICROBIOLOGICAL METHOD OF β-ALANINE ASSAY

By JAY R. SCHENCK*

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, May 6, 1943)

The existence of β-alanine in muscle tissue has been recognized since its discovery in muscle extract by Engeland (1) and its identification as a component of carnosine by Gulewitsch (2). β-Alanine early attracted speculation as to its origin, since, in contrast to most of the other known amino acids found in nature, its amino group is in the β rather than in the α position. The conversion of aspartic acid to β-alanine by putrefactive bacteria was demonstrated by Ackermann (3) and a similar reaction has been postulated for the formation of carnosine in the animal body. A hypothetical precursor, β-aspartylhistidine, has been assumed to undergo decarboxylation to produce carnosine (β-alanylhistidine).



As yet, no experimental evidence has been brought forth to confirm this hypothesis. Du Vigneaud and Hunt (4) synthesized β-aspartylhistidine and showed that it, like carnosine, could support growth of rats on a diet deficient in histidine. This finding justified attempts to establish experimental conditions which would test whether or not the conversion occurred.

For such experiments, a sensitive method was needed for the detection of carnosine or β-alanine. Most of the existing methods of analysis for carnosine depended on a colorimetric reaction of the histidine portion of the carnosine molecule, and since aspartylhistidine would interfere, it was necessary to find a more specific test for carnosine. The discovery of Mueller (5, 6) that β-alanine is an essential growth factor for the diphtheria bacillus provided the means of developing a sensitive and specific test for this compound. By the use of this test, carnosine and β-alanine could be

* Abbott Laboratories Research Fellow.

detected in the presence of aspartic acid or aspartylhistidine, and it was possible to devise a quantitative method for the determination of the concentration of β-alanine. The total bacterial growth permitted by an unknown sample was compared with that produced by known amounts of pure β-alanine. Since Mueller (7) had shown that carnosine was less effective than β-alanine in stimulating growth, it was decided at the outset to hydrolyze all samples to be assayed. A typical standard curve of bacterial growth as a function of β-alanine content of the medium is shown in Fig. 1.

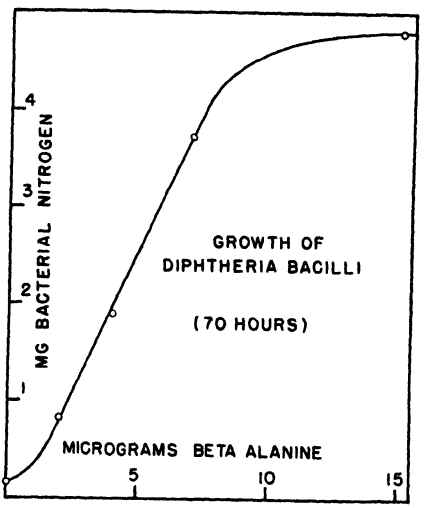


Fig. 1. Influence of amount of β-alanine on the total growth of diphtheria bacilli

The specificity of this method has been tested with a group of compounds related to β-alanine. Among the compounds incapable of replacing β-alanine in the growth test are anserine, *d*-carnosine, β-alanylhistamine, β-aminobutyric acid, and β-aminoisobutyric acid.

The growth test requires about 5 γ of β-alanine, an amount which compares favorably with the most sensitive chemical method for carnosine which requires about 50 γ (8). An independent method based on the stimulation of yeast growth by β-alanine, which will be described in detail in a subsequent publication, checked very well with the bacterial growth method.[1]

With the development of a satisfactory method for the assay of carnosine, it became possible to do experiments bearing on the postulated conversion

[1] Since the preparation of this manuscript, a note by Pollack (9) has appeared, describing the use of a yeast growth method for β-alanine assay.

of aspartylhistidine to carnosine. Two different methods of approach were used. The first was the incubation of tissue slices in the presence of aspartylhistidine or aspartic acid. No increase in β-alanine was observed during the incubation of these compounds with kidney, liver, or muscle tissue. Control experiments showed that neither β-alanine nor carnosine was destroyed under the conditions employed.

The second method involved the use of the whole animal. Preliminary experiments showed that after injection, β-alanine and carnosine disappeared slowly from the blood stream of rabbits. On the basis of this observation, it was thought possible that β-alanine might be found in the blood or urine after the injection of aspartylhistidine and aspartic acid if they were decarboxylated rapidly in the intact animal. The growth test for β-alanine was sufficiently sensitive so that the appearance of 1 per cent of the injected compound as β-alanine in the blood or urine could have been detected. However, no indication of a conversion was obtained by this method. Thus the experimental results obtained do not afford any support for the hypothetical conversion of aspartylhistidine to carnosine.

EXPERIMENTAL

β-Alanine Assay Method

A culture of the Allen strain of *Corynebacterium diphtheriae* was carried with daily transfers on the following medium[2] (in mg. per liter of solution): casein hydrolysate 1000, *l*-cystine 10, *l*(+)-glutamic acid 500, glycerol 500, β-alanine 0.5, nicotinic acid 0.5, pimelic acid 0.06, Na_2HPO_4 240, KH_2PO_4 42, MgO 6.4, $FeSO_4 \cdot 7H_2O$ 1.0, $MnCl_2 \cdot 4H_2O$ 0.5, $CuSO_4 \cdot 5H_2O$ 1.0, $ZnSO_4 \cdot 7H_2O$ 1.8, $CaCl_2 \cdot 6H_2O$ 10. The solution was neutralized to pH 7.5. The hydrolyzed casein was prepared by boiling casein with 20 per cent HCl for 18 hours and the excess acid was removed by repeated evaporation under reduced pressure. Calcium salts were removed by making the hydrolysate alkaline.

The basal medium for the assay of β-alanine, essentially that of Mueller (10), contained (in mg. per liter of solution): HCl-hydrolyzed casein 10,000, *l*-aspartic acid 1250, *l*(+)-glutamic acid 1250, cystine 600, KCl 400, Na_2HPO_4 3000, $MgCl_2$ 200, pimelic acid 0.15, nicotinic acid 2.3, ethyl alcohol 7000, lactic acid 17,500, $CaCl_2 \cdot 6H_2O$ 200, $FeSO_4 \cdot 7H_2O$ 5, $MnCl_2 \cdot 4H_2O$ 2.5, $CuSO_4 \cdot 5H_2O$ 5, $ZnSO_4 \cdot 7H_2O$ 9. A stock solution of this medium was maintained with all the ingredients at 3 times the above concentration except cystine, calcium chloride, and magnesium chloride. Just before use, the cystine was added and the medium was diluted to four-fifths the final volume and was adjusted to pH 7.5 (phenol red). 8 ml. of this

[2] The author is indebted to Professor J. H. Mueller of Harvard Medical School for the culture and for formulae of the media.

medium were placed in a test-tube together with 2 ml. of a neutral sample to be assayed. The tubes were stoppered with cotton plugs and autoclaved for 10 minutes at 10 pounds pressure. A sterile solution of the calcium and magnesium salts was then added, followed by a loopful of the pellicle from a day-old culture of diphtheria bacilli. The mixture in the tubes was incubated in a slanting position for about 70 hours (maximum growth is attained in this time). After incubation, the total nitrogen content of the bacterial cells was determined in a manner described by Mueller (11). The β-alanine content was determined by comparing the growth given by the unknown sample with a standard curve obtained with pure β-alanine in amounts varying from 0 to 15 γ as shown in Fig. 1. In the assays carried out by this method, duplicates performed on different days checked within 30 per cent.

The unknown samples were prepared by hydrolysis with 10 per cent hydrochloric acid. For small portions of tissue (50 to 500 mg.), it was convenient to seal the acid and the tissue in a test-tube and to heat in an oil bath at 100–135° for 20 hours. The hydrolysate could be neutralized with strong sodium hydroxide and was ready for assay after the humin was removed by filtration. Added β-alanine could be completely recovered from tissues hydrolyzed in this manner.

Tissue Experiments

The following is a typical experiment with rat kidney. The kidneys (3 gm.) from an adult male rat which had been killed by a blow on the head were decapsulated and part of the medulla was removed. The cortex was cut into small pieces and homogenized, together with 6 ml. of Krebs-Ringer-phosphate solution. 2 ml. of this homogenized suspension were placed in Thunberg tubes which contained aspartylhistidine or aspartic acid in the side arm. The tubes were evacuated and the substrate was added to the tissue by tipping the tube. The tubes were incubated at 37.5° for 2 hours, and the contents were assayed for β-alanine after hydrolysis. No production of β-alanine could be detected. The same results were obtained with rat liver. Muscle strips incubated aerobically in Ringer-bicarbonate-glucose solution also yielded negative results. Experiments with slices of rat liver and kidney and minced muscle tissue incubated in Ringer-phosphate solution in the Warburg manometric apparatus showed no increased carbon dioxide production in the presence of aspartylhistidine after approximately 4 hours of incubation. The increase found after this period was attributed to bacterial action and no β-alanine was found by assay. Controls for the above experiments showed that carnosine was not destroyed under the conditions used.

Intact Animal Experiments

Injection of β-Alanine or Carnosine—In a typical experiment, 650 mg. of β-alanine were injected into a male rabbit weighing 2.2 kilos. After 20, 67, and 212 minutes, the blood contained 0.9, 0.27, and 0.03 mg. of β-alanine per ml. respectively. In 1 hour about 5 per cent of the injected β-alanine appeared in the urine.

Injection of Aspartylhistidine or Aspartic Acid—390 mg. of aspartylhistidine were injected into the marginal ear vein of a male rabbit weighing 1.85 kilos. Blood samples removed after 14 and 60 minutes contained no β-alanine. If 1 per cent of the injected aspartylhistidine had appeared in the blood as carnosine, it could have been detected. The urine contained no more β-alanine after the injection than normal urine, and again an excretion of 1 per cent of the theoretical β-alanine could have been detected. Several other similar experiments and one with aspartic acid also gave negative results.

SUMMARY

A microbiological assay method for β-alanine based on the growth of the diphtheria bacillus is described. This method has been used as an analytical method in tissue experiments and experiments with intact animals, devised to test hypotheses regarding the origin of β-alanine in the animal organism. From these experiments, designed to investigate the possible conversion of aspartic acid to β-alanine or of aspartylhistidine to carnosine, no evidence was found to support the hypothesis that β-alanine is derived from aspartic acid or carnosine from aspartylhistidine.

The author wishes to express his appreciation to Professor Vincent du Vigneaud for his suggestion of the problem and for his interest and counsel throughout the course of the investigation. The author is also indebted to Dr. Dean Burk, Dr. Fritz Lipmann, and Dr. William H. Summerson for advice regarding the experiments in the Warburg apparatus.

BIBLIOGRAPHY

1. Engeland, R., *Z. Untersuch. Nahrungs- u. Genussmittel*, **16**, 658 (1908).
2. Gulewitsch, W., *Z. physiol. Chem.*, **73**, 434 (1911).
3. Ackermann, D., *Z. Biol.*, **56**, 87 (1911).
4. du Vigneaud, V., and Hunt, M., *J. Biol. Chem.*, **125**, 269 (1938).
5. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, **36**, 706 (1937).
6. Mueller, J. H., and Cohen, S., *J. Bact.*, **34**, 381 (1937).
7. Mueller, J. H., *J. Biol. Chem.*, **123**, 421 (1938).
8. Eggleton, M., and Eggleton, P., *Quart. J. Exp. Physiol.*, **23**, 391 (1933).
9. Pollack, M. A., *J. Am. Chem. Soc.*, **65**, 484 (1943).
10. Mueller, J. H., *J. Bact.*, **36**, 499 (1938).
11. Mueller, J. H., *J. Bact.*, **29**, 383 (1935).

STUDIES ON KETOSIS

XXI. THE COMPARATIVE METABOLISM OF THE HEXITOLS*

By CORNELIA JOHNSTON and HARRY J. DEUEL, Jr.

(From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles)

(Received for publication, March 1, 1943)

The ready availability of the hexitols due to the development of methods of synthesis by electrolytic reduction of the corresponding hexoses has made a more complete understanding of their metabolic fate desirable. There seems to be some evidence that their metabolism resembles that of the analogous sugars.

Sorbitol is the most readily available physiologically of the hexitols. Although Payne, Lawrence, and McCance (1) were unable to demonstrate an increase in the liver glycogen of rats after the oral administration of sorbitol, Waters (2) showed that marked glycogen deposition resulted when the material was injected intraperitoneally, a result which was confirmed by Todd, Myers, and West (3). After oral administration, Todd, Myers, and West (3), Carr and Forman (4), and Blatherwick *et al.* (5) demonstrated some degree of glycogen deposition in the liver. Better results were obtained if the sorbitol was fed in cacao butter. This probably lessens peristalsis and prevents the diarrhea which usually accompanies the ingestion of large quantities of aqueous solutions of sorbitol.

Mannitol is much less readily metabolized. Silberman and Lewis (6) were unable to demonstrate the conversion of mannitol to glycogen when fed in aqueous solution to rats. When it is fed as 33 per cent of cacao butter diet, Carr *et al.* (7) found some glycogen storage, although its metabolism was at too slow a rate to have any effect on the respiratory quotient. Carr and Krantz (8) and Todd, Myers, and West (3) also found glycogenesis after the cacao butter-mannitol mixture was given, although the last workers found no response when the hexitol was administered alone orally or intraperitoneally.

Dulcitol was found by Carr and Krantz (9) to be a source of liver glycogen in rats and rabbits when fed in cacao butter, although no noticeable effect on the R.Q. of rats or on the blood sugar of rabbits could be noted.

Because of the contradictory results which have been obtained, it seemed

* Some of the data reported here were presented at a meeting of the Southern California Section of the Society for Experimental Biology and Medicine held at the California Institute of Technology, June, 1941. They were presented by Cornelia Johnston in partial fulfilment of the degree of Master of Science in Biochemistry in the Graduate School of the University of Southern California.

TABLE I

Liver and Muscle Glycogen of Normal Rats; Previously Fasted 48 Hours, at Various Periods after Intraperitoneal Injections of Hexitols, Glucose, or Sodium Chloride

Substance injected*	Sex	Body weight	Liver				Muscle			
			No. of experiments	Glycogen†	Fisher *t*		No. of experiments	Glycogen†	Fisher *t*	
					Calculated‡	Found			Calculated‡	Found
6 hrs. after injection										
		gm.		*per cent*				*per cent*		
NaCl. ..	M.	146	15	0.21 ± 0.03			5	0.26 ± 0.04		
Glucose ..		154	15	3.18 ± 0.20	2.76	12.98	5	0.59 ± 0.04	3.36	5.73
Sorbitol.....		149	15	3.75 ± 0.20	2.76	16.40	4	0.34 ± 0.02	3.50	1.54
					2.76§	1.96§			3.50§	5.05§
Mannitol.		151	14	0.47 ± 0.09	2.76	2.70	6	0.25 ± 0.02		
NaCl .		222	5	0.22 ± 0.08			3	0.23 ± 0.03		
Glucose‖ ..		227	10	1.53 ± 0.12	3.01	6.75	8	0.34 ± 0.02	3.25	3.17
Dulcitol‖		224	10	0.30 ± 0.06	3.01	0.73	7	0.23 ± 0.02		
NaCl. . .	F.	108	9	0.08 ± 0.06			7	0.21 ± 0.03		
Glucose....		111	9	1.97 ± 0.15	2.92	11.15	9	0.37 ± 0.02	2.80	6.12
Sorbitol..		112	10	2.35 ± 0.01	2.90	13.88	9	0.30 ± 0.01	2.98	3.53
					2.90§	1.77§			2.92§	2.90§
Mannitol.		106	10	0.13 ± 0.05	2.90	0.61	5	0.28 ± 0.07	3.17	1.02
12 hrs. after injection										
NaCl .	F.	136	10	0.01 ± 0.01			9	0.22 ± 0.02		
Glucose . ..		130	9	1.81 ± 0.27	2.90	6.37	9	0.43 ± 0.02	2.92	6.46
Sorbitol..		130	10	1.87 ± 0.15	2.88	11.32	9	0.40 ± 0.03	2.92	5.95
18 hrs. after injection										
NaCl	F.	139	10	0.04 ± 0.02			9	0.20 ± 0.02		
Glucose		133	10	1.88 ± 0.22	2.88	7.71	10	0.37 ± 0.03	2.90	4.82
Sorbitol		136	9	2.21 ± 0.12	2.90	18.20	9	0.37 ± 0.03	2.92	4.56
					2.92§	1.19§				

* The strength of the glucose and hexitol solutions was 25 per cent unless otherwise noted, while that of sodium chloride was 0.9 per cent. In the experiments on males, 1.0 cc. per 100 sq. cm. of surface area was used; in the females the dose was only 0.5 cc. per 100 sq. cm.

† Including the standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where d is the deviation and n is the number of experiments.

‡ Based on a p value of 0.01 (1 chance in 100 that the difference is due to experimental error). See Fisher (14).

§ Comparison with glucose; other comparisons are with controls which received sodium chloride. In both series of 6 hour experiments, the liver glycogen after sorbitol is significantly higher than after glucose if a p value of 0.1 is used.

‖ 9 per cent soultion injected.

desirable to follow the level not only of liver glycogen, but also of muscle glycogen after intraperitoneal injection of glucose and the hexitols; also a comparison of the ability of the sugar alcohols to be changed to glucose seemed worth while by other means of evaluation, such as the degree to which they lower exogenous and endogenous ketonuria. Finally, because of some unexpected results obtained with sorbitol when administered to rats having an endogenous ketonuria, comparisons were also made of glycogenesis in animals having fatty livers.

TABLE II

Comparative Effect of Glucose and Various Hexitols on Exogenous Ketonuria of Fasting Female Rats Fed 150 Mg. of Sodium Butyrate (As Acetone) per 100 Sq. Cm. in Two Divided Doses Daily

Substance fed	Body weight at start	Acetonuria per 100 sq. cm. as acetone*						
		1st day	2nd day	3rd day	4th day	Average of 4 days		
						Value	$\frac{\text{M D}}{\text{S.E M D.}}$ †	Fisher *t* found (calculated, 2.58‡)
	gm.	*mg*	*mg.*	*mg*	*mg*	*mg*		
Controls	141	105.4 ± 5.7 (11)	106.1 ± 5.4 (11)	108.9 ± 4.9 (9)	98.2 (1)	106.5 ± 2.9 (32)		
Glucose	148	55.8 ± 7.5 (8)	47.8 ± 5.9 (8)	37.5 ± 7.3 (6)		47.9 ± 4.3 (22)	11.32	11.20
Sorbitol	151	87.0 ± 6.4 (10)	73.3 ± 3.8 (9)	72.8 ± 4.0 (7)	55.7 (2)	76.9 ± 3.3 (28)	6.53	6.53
Dulcitol	135	88.7 ± 7.4 (8)	89.4 ± 8.4 (8)	96.1 ± 7.9 (7)	82.4 (2)	90.5 ± 4.1 (25)	3.19	3.14
Mannitol	157	99.9 ± 8.1 (12)	91.7 ± 4.9 (12)	90.0 ± 7.7 (8)	73.7 (1)	93.9 ± 3.9 (33)	2.57	2.51

The figures in parentheses represent the number of determinations in the average.

* Including the standard error of the mean, calculated as in Table I.

† Ratio of the mean difference to the standard error of the mean difference. When this exceeds 3.00, the value is considered statistically significant as compared with controls.

‡ Based on a *p* value of 0.01 (1 chance in 100 that the difference is due to experimental error).

EXPERIMENTAL

In the studies on glycogenesis, the hexitols, glucose, and sodium chloride were administered intraperitoneally in two doses at 3 hour intervals to rats previously fasted for 48 hours. The total amount injected per 100 sq. cm. of body surface was 1 cc. of a 25 per cent glucose, sorbitol, or mannitol solution or of a 9 per cent glucose or dulcitol solution or physiological saline. The dulcitol solution was a saturated one. The gastrocnemius muscle and

liver were removed under sodium amytal anesthesia. After the muscle was exposed,[1] the whole area was frozen *in situ* with an ether-CO_2 mixture and then removed. The liver was also immediately removed, frozen in a dry ice-ether mixture, and weighed and the glycogen determined by the procedure of Good, Kramer, and Somogyi (10).

TABLE III

Comparative Effect of Glucose and Various Hexitols on Endogenous Ketonuria of Fasting Female Rats Fed 25 Mg. per 100 Sq. Cm. Twice Daily

Substance fed	Body weight	Acetonuria per 100 sq cm. as acetone*					Urine N	Liver analyses	
		2nd day	3rd day	4th day†	Average	M.D./S.E.M.D. ‡		Water	Lipid
	gm.	mg.	mg.	mg.	mg.		mg. per 100 sq. cm	per cent	per cent
Controls	138	31.7 ± 4.2 (13)	39.1 ± 3.1 (13)	35.8 ± 3.2 (9)	35.5 ± 2.2 (35)		28.3	54.4	28.4
Glucose	135	17.3 ± 3.0 (12)	19.4 ± 2.8 (14)	10.8 ± 2.5 (10) **6.17**	16.3 ± 1.7 (36)	6.85	25.8	55.2	27.8
Sorbitol	139	25.2 ± 3.0 (14)	36.9 ± 3.5 (14)	29.4 ± 4.0 (14) **1.25**	30.5 ± 2.2 (42)	1.61	22.6	59.6	20.3
Dulcitol	135	31.5 ± 4.0 (14)	39.8 ± 2.8 (14)	21.6 ± 2.9 (11) **3.28**	31.7 ± 2.2 (39)	1.22	27.3	55.3	26.8
Mannitol	135	31.1 ± 3.9 (13)	31.7 ± 2.6 (12)	19.6 ± 4.0 (10) **4.00**	28.1 ± 2.2 (35)	2.39	25.5	58.9	20.1

The figures in parentheses represent the number of experiments in the average.

* Including the standard error of the mean, calculated as in Table I.

† The figures in bold-faced type are the ratio of the mean difference to the standard error of the mean difference for the 4th day only when compared with the controls.

‡ Ratio of the mean difference to the standard error of the mean difference. When this exceeds 3.0, the results are considered significant.

Exogenous ketonuria was produced by the oral administration twice daily of 0.5 cc. of a 22.5 per cent solution of sodium butyrate (containing 150 mg. of butyric acid calculated as acetone) per 100 sq. cm. of surface area, as employed earlier (11). The hexitols and glucose were administered simultaneously in an amount of 25 mg. per 100 sq. cm. twice daily. In order to produce an endogenous ketonuria, fatty livers were first developed

[1] Only those muscles were analyzed in which no contraction occurred prior to immersion in the freezing mixture.

in rats by feeding a high fat diet containing Crisco over a 12 day period, as in earlier work (12). On subsequent fasting, the effect of administration of 25 mg. of glucose twice daily or equivalent weights of the hexitols on the resulting ketonuria was followed.

At the conclusion of the experiments on endogenous ketonuria, the animals were sacrificed and the water and lipid contents of the liver were determined (12). In the results recorded in Table IV, glycogen was also determined as a portion of the liver.

The rats used were from our stock colony. In each series of experiments litter mates were equally distributed among the different groups as far as

TABLE IV

Liver Glycogen of Fat-Free Liver 6 Hours after Intraperitoneal Injection of Glucose or Sorbitol in Female Rats Previously Fasted 2 Days after Having Received High Fat, Low Protein Diet for 12 Days

Liver lipid	Glucose					Sorbitol				
	No. of experiments	Body weight*	Liver glycogen on fat-free basis			No. of experiments	Body weight*	Liver glycogen on fat-free basis		
			Maximum	Minimum	Mean			Maximum	Minimum	Mean
per cent		*gm.*	*per cent*	*per cent*	*per cent*		*gm.*	*per cent*	*per cent*	*per cent*
<5	3	146	3.34	0.61	1.98					
5.00– 9.99	7	133	1.68	0.41	1.22	7	134	2.66	0.62	1.62
10.00–14.99	5	127	1.82	0.32	1.00	9	130	2.12	0.64	1.38
15.00–19.99	7	128	1.07	0.50	0.80	5	154	2.26	1.56	1.78
20.00–24.99	7	125	1.56	0.26	1.05	3	124	1.66	0.46	1.19
25.00–29.99	6	107	2.00	0.00	1.01	5	123	1.45	0.39	0.91
30.00–34.99	4	103	2.42	0.21	1.14	7	118	1.70	0.38	0.95
35.00–44.99	5	106	2.31	0.31	1.05	6	116	1.47	0.34	0.91

* At the time of injection, after a 48 hour fast.

possible. The hexitols were highly purified products from the following sources: sorbitol (Atlas Powder Company), mannitol (Pfanstiehl), and dulcitol (Merck). Surface area was calculated by the formula of Lee (13).

Results

The average liver and muscle glycogen of the rats receiving the various hexitols, glucose, and physiological saline solution are given in Table I.

A comparison of the effect of glucose and the hexitols on the exogenous ketonuria produced by the feeding of sodium butyrate is given in Table II. Table III presents a similar summary on rats having an endogenous ketonuria produced by the previous feeding of a high fat, low protein diet.

Table IV records the values of liver glycogen in female rats in which fatty

livers had been developed by a high fat, low protein diet, as described earlier (11), 6 hours after the intraperitoneal injection of glucose or sorbitol. The content of glycogen is calculated on the basis of a fat-free liver so that it may be more readily compared with the glycogen levels, recorded in Table I, of normal fat levels in the livers. The data demonstrate that a marked reduction in ability to form liver glycogen occurs in animals with fatty livers; moreover, this decreased transformation is more evident after sorbitol than after glucose.

DISCUSSION

In each of the four series of experiments carried out to compare the glycogenic ability of glucose and sorbitol, which are recorded in Table I (6 hours after administration on male and female rats, 12 and 18 hours on females), the average level of liver glycogen was invariably slightly higher in the sorbitol-injected rats. In the 6 hour experiments, the liver glycogen after sorbitol and glucose averaged 3.75 and 3.18 per cent respectively in the males and 2.35 and 1.97 per cent in the females. Approximately equal levels were obtained at 12 hours (1.87 and 1.81 per cent respectively), although the levels for sorbitol again were higher at 18 hours (2.21 and 1.88 per cent).

On the other hand, the injection of glucose caused a much more immediate marked rise in muscle glycogen than did sorbitol. In the 6 hour experiments on males, the muscle glycogen in the rats receiving sorbitol had been elevated only from the control level of 0.26 to 0.34 per cent, while in those which were given glucose, the value was 0.59 per cent. Similar results were obtained in the 6 hour experiments on females (control 0.21, sorbitol-fed 0.30, glucose-fed 0.37), while at 12 and 18 hours the rats receiving sorbitol and glucose had practically identical levels of muscle glycogen.

After the injection of mannitol a slight increase in liver glycogen was noted in the 6 hour experiments on males. The rise in liver glycogen was far less than that reported by Deuel, Hallman, Murray, and Hilliard (15) after the intraperitoneal injection of mannose, when an average value of 1.28 per cent was found. Dulcitol caused practically no increase in liver or muscle glycogen, in contrast to the much greater effect of galactose (16). The glycogen present in the muscles as well as the livers of the control rats was invariably higher in the male rats than in the female rats, which confirms our earlier findings (17).

On the basis of the studies of ketonuria, however, the ability of sorbitol to be converted to glucose is much less than would be expected from the results on liver glycogen. The administration of sorbitol caused an average lowering of ketone bodies of 29.6 mg. (from the control level of 106.5 to

76.9 mg.), while a similar dose of glucose brought about twice the decrease (58.6 mg.). The drop in ketonuria after dulcitol and mannitol was much less (16.0 and 12.6 mg. respectively) but both were significantly lower than the control level.

On the other hand, sorbitol produced an effect which was only 25 per cent that of glucose in the experiments in which an endogenous type of ketonuria was employed. Whereas the lowering of ketonuria after the daily administration of 50 mg. of glucose was 19.2 mg., the decrease with sorbitol was only 5 mg. This value was no greater than with dulcitol and mannitol. With the latter hexitols, however, the effect was only noted on the 3rd and 4th days. Possibly some cumulative effect may have occurred here.

In our earlier studies we have usually found a correlation between glycogenesis and ketolytic activity. This is certainly not evident in the present experiments. Possibly the comparison should rather be made between muscle glycogen and ketolysis. It is in the muscle that the ketone bodies are oxidized as well as glucose. If any such relationship exists between the disappearance of these substances, it should be related to their concentration at the site of oxidation.

Another explanation is needed for the lowered activity of sorbitol in endogenous compared with exogenous ketonuria. This can readily be explained if the transformation of sorbitol to glucose is interfered with in the animals with fatty livers due to liver damage, since it appears that the liver is the chief site for oxidation of sorbitol to glucose. If glycogen were not formed in the liver, it could not be available for muscle glycogen. That such damage may occur is suggested by other experiments in which a decreased glucose tolerance was noted in rats with fatty livers, owing presumably to decreased ability in glycogenesis (18). In the experiments with animals having fatty livers, it was found that the level of liver glycogen was markedly lower after the administration of glucose or sorbitol than in rats in which the lipid content of the liver was normal. The reduction in glycogenesis was greater with sorbitol than with glucose. However, since glucose is capable of being transformed directly to muscle glycogen without any preliminary alteration in the liver, while apparently this cannot occur with sorbitol, the discrepancy in the ketolytic action here becomes greater.

SUMMARY

Sorbitol was found to act slightly more effectively than glucose in causing a deposition of liver glycogen when injected intraperitoneally in male or female rats in 6 hour studies and in female rats in the 12 to 18 hour experiments. However, glucose differs in causing a much more prompt and greater increase in muscle glycogen, which is evident in the 6 hour experi-

ments. The maximum muscle glycogen after sorbitol administration was not found until 12 hours later; after 18 hours the levels of muscle glycogen were still elevated over the control values in both groups and they were identical. However, the ketolytic effect of sorbitol was only 50 per cent that of glucose, as shown by exogenous ketonuria; in experiments in which an endogenous ketonuria was employed, sorbitol was shown to have only 25 per cent the effectiveness of glucose. Because the level of glycogen is somewhat higher in the liver and much lower in the muscle after sorbitol than after glucose, it is suggested that the liver is probably the site of transformation of sorbitol to glucose. The lower effect of sorbitol in endogenous compared with exogenous ketonuria is believed to be caused by the failure of the fatty liver to convert sorbitol to glycogen at its normal rate, owing to a derangement in its function in animals with fatty livers.

There is evidence from experiments with exogenous ketonuria that mannitol and dulcitol are both ketolytic agents and therefore must be potential sources of glucose. They are only half as effective as sorbitol and 25 per cent as effective as glucose. In the studies on glycogen formation, mannitol was found to be only weakly active, while no proof of the transformation of dulcitol to this polysaccharide could be noted.

BIBLIOGRAPHY

1. Payne, W. W., Lawrence, R. D., and McCance, R. H., *Lancet*, **2**, 1257 (1933).
2. Waters, E. T., *Proc. XVI Internat. Physiol. Cong.*, Zurich, 122 (1938).
3. Todd, W. R., Myers, J., and West, E. S., *J. Biol. Chem.*, **127**, 275 (1939).
4. Carr, C. J., and Forman, S. E., *J. Biol. Chem.*, **128**, 425 (1939).
5. Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., Larson, H. W., and Sawyer, S. D., *J. Biol. Chem.*, **134**, 549 (1940).
6. Silberman, A. K., and Lewis, H. B., *Proc. Soc. Exp. Biol. and Med.*, **31**, 253 (1933–34).
7. Carr, C. J., Musser, R., Schmidt, J. E., and Krantz, J. C., Jr., *J. Biol. Chem.*, **102**, 721 (1933).
8. Carr, C. J., and Krantz, J. C., Jr., *J. Biol. Chem.*, **124**, 221 (1938).
9. Carr, C. J., and Krantz, J. C., Jr., *J. Biol. Chem.*, **107**, 371 (1934).
10. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
11. Deuel, H. J., Jr., Hallman, L. F., and Murray, S., *J. Biol. Chem.*, **124**, 385 (1938).
12. Deuel, H. J., Jr., and Hallman, L. F., *J. Biol. Chem.*, **140**, 545 (1941).
13. Lee, M. C., *Am. J. Physiol.*, **89**, 24 (1929).
14. Fisher, R. A., Statistical methods for research workers, London, 6th edition (1936).
15. Deuel, H. J., Jr., Hallman, L. F., Murray, S., and Hilliard, J., *J. Biol. Chem.*, **125**, 79 (1938).
16. Deuel, H. J., Jr., MacKay, E. M., Jewel, P. W., Gulick, M., and Grunewald, C. F., *J. Biol. Chem.*, **101**, 301 (1933).
17. Deuel, H. J., Jr., Gulick, M., Grunewald, C. F., and Cutler, C. H., *J. Biol. Chem.*, **104**, 519 (1934).
18. Deuel, H. J., Jr., and Davis, A., *J. Biol. Chem.*, **146**, 649 (1942).

CHEMICAL AND BIOLOGICAL STABILITY OF CRYSTALLINE VITAMINS D_2 AND D_3 AND THEIR DERIVATIVES

By W. HUBER and O. W. BARLOW

(From the Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, New York)

(Received for publication, April 6, 1943)

Fritz, Halpin, Hooper, and Kramke (1) presented evidence that vitamin D is not stable when added to various dry carriers as it must be added of necessity to mixed feeds. The carriers used include dried whey, sucrose, oyster shell, mineral mixtures, sand, kaolin, and $MnSO_4$ in mixtures with such solvents as ether, corn oil, tuna oil, and the U.S.P. reference oil with a menstruum such as cereals and ground corn. Instability is due to numerous substances or conditions that promote oxidation. Temperature coefficients and other data indicate that the destruction is due to a chemical reaction.

Thus, all forms of vitamin D, including activated ergosterol in a crystalline form or when adsorbed on casein (ertron), activated 7-dehydrocholesterol (crystalline vitamin D_3), as well as vitamin D from natural sources, are susceptible to destruction. This change occurs more rapidly in a dry dispersed state than in emulsions.

Various methods for stabilizing vitamin D have been tested. The most effective protection is afforded by coatings which prevent contact with air. Thus, coating agents such as dried whey with hydrogenated fats and calcium stearate impart materially more stability than does whey alone. Mineral mixtures deteriorated rapidly, but were satisfactorily stabilized by hydrogenated fats or even molasses. Packing in inert gases additionally retards the rate of destruction.

Baird, Ringrose, and MacMillan (2) reported that the vitamin D content of chick ration fortified with cod liver oil and stored at summer temperatures for 32 weeks showed no measurable destruction. Jung (3) noted that peanut oil solutions of vitamin D_3 were less stable than similar solutions of vitamin D_2, but the stability on addition of stabilizing substances such as yeast, alfalfa meal, and $CaCO_3$ was satisfactory.

The present report deals with the stability of crystalline D vitamins and their derivatives *per se*, as well as when incorporated in pharmaceutical preparations.

During the last 4 years large amounts of vitamins D_2 and D_3 and their derivatives have gone through our hands and we have tried to establish their data in terms of melting point, specific rotation, and ultraviolet ab-

TABLE I

Melting Points, Specific Rotation, and Ultraviolet Absorption of Vitamin D_2 and D_3 Esters

Vitamin	Ester	M.p.	Specific rotation		Ultraviolet absorption*		Appearance	Color
			$[\alpha]_D^{20}$	1.6 per cent solution	Maximum	$E \times 10^{-3}$		
		°C.	*degrees*		λ			
D_2	4-Nitrobenzoate (4)†	94.5– 95	+105.2 +107.4	Chloroform Acetone	261	30.8 ± 0.4	Sturdy prisms	Bright yellow
	3,5-Dinitrobenzoate (5)	146 –147	+91.5 +86.5	Chloroform Acetone	265‡	22.5 ± 0.4	Prisms, from acetone. Needles, from chloroform-ethanol	Canary "
	4-Methyl-3-nitrobenzoate§	119 –120	+106.8 +105.0	Chloroform Acetone	261	22.2 ± 0.4	Needles	Pale yellow
	4-Methyl-3,5-dinitrobenzoate (6)	116 –117	+95.8 +92.2	Chloroform Acetone	265‡	21.0 ± 0.4	Fine needles from hexane	Light "
D_3	4-Nitrobenzoate (4)	125 –126	+114.6 +116.4	Chloroform Acetone	261	30.8 ± 0.4	Prisms	Very light yellow
	3,5-Dinitrobenzoate (4)	132 (Benzol-methanol) 141 (Ether)	+97.9 +96.3	Chloroform Acetone	265‡	22.8 ± 0.4	Fine needles	Cream
	4-Methyl-3,5-dinitrobenzoate§	128–129	+106.6 +101.2	Chloroform Acetone	265‡	20.8 ± 0.4	Very fine needles from acetone	Light yellow

* The absorption spectra were taken by Dr. G. W. Ewing in our laboratories with a Beckman quartz spectrophotometer, model DU.

† The figures in parentheses represent bibliographic references.

‡ This is not a true maximum but an arbitrary identification value on the slope of the absorption curve, the true maximum being outside the range of our instrument.

§ These are new compounds. Their preparation and their analytical data will be published shortly in the section "New compounds" of the *Journal of the American Chemical Society*.

sorption with the greatest possible accuracy. The esters of vitamins D_2 and D_3 with various nitrobenzoic acids are not only characteristic derivatives, but are also optimally suited for storage purposes from the standpoint of stability. They remain unchanged over a period of years if a slight photodecomposition is avoided by storage in amber bottles.

It is our experience that pure crystalline vitamins D should be prepared by direct saponification of rigidly purified esters rather than by recrystallization. The latter procedure is always accompanied to some extent by more or less decomposition based on determinations of the physical constants.

The data for melting point, specific rotation, and ultraviolet absorption on the 4-nitrobenzoates, 3,5-dinitrobenzoates, and 4-methyl-3,5-dinitrobenzoates of vitamins D_2 and D_3 are incomplete and widely scattered over

TABLE II

Melting Points, Specific Rotation, and Ultraviolet Absorption of Pure Crystalline Vitamins D_2, D_3, and D_3-Cholesterol

Substance	M.p.	Specific rotation		$E \times 10^{-3}$ (maximum ultraviolet absorption, 265 λ)	Appearance (color, white)
		$[\alpha]_D^{20}$	1 6 per cent solution		
	°C.	*degrees*			
Vitamin D_2	116–117*	+48.2	Chloroform	19.50–19.70	Fine clustered needles
		+83.4	Acetone		
" D_3	84– 85*	+51.9	Chloroform	18.90–19.20	Needles
		+84.8	Acetone		
" D_3-cholesterol	119–120*	+26.4	"	9.45– 9.60	Fine clustered needles

* The sample was dropped into a bath 15° below the melting point. The rate of heating was 6–7° per minute.

the literature. Table I shows the physicochemical data of these esters and of the 4-methyl-3-nitrobenzoates of vitamins D_2 and D_3, which in our experience are characteristic for the pure compounds.

Anderson, Bacharach, and Smith (7) have investigated extensively the properties of crystalline vitamin D_2. We are able to confirm their values, although in our experience the $E_{1\,cm.}^{1\%}$ values are not scattered over quite such a wide range. Table II shows data which in our experience are the constants in terms of melting point, specific rotation, and ultraviolet absorption of pure crystalline vitamins D_2 and D_3 (8) and the vitamin D_3-cholesterol double compound. The latter (9) is an addition product or compound of vitamin D_3 and cholesterol in molecular proportions. It can be obtained from crude vitamin D_3 resins in over 90 per cent yield and thus represents the most economical way to obtain a crystalline vitamin D_3

TABLE III

Stability of Vitamin D Preparations

	Evacuated brown ampules stored at +4°					Brown ampules stored in air at +4°				Brown bottles stored in air at +25°				
	3 mos.	6 mos.	9 mos.	12 mos.	18 mos.	1 wk.	2 wks.	3 wks.	6 wks.	10 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
Vitamin D_2														
M.p., °C....	116–117	116	116	115–116	113–114	116	115.5–116	113–114	109–110	116–117	116	114–115	110–113	107–109
$[\alpha]_D^{20}$ (1% acetone), *degrees* ..	+82.9	+83	+82.7	+82	+81.6	+82.7	+82.3	+81.6	+78.4	+82.9	+82.8	+82	+79.6	+79.1
Vitamin D_3														
M.p., °C....	84.5	85	84.5	84.5	80	84.5		84.5	73–75	84.5			79–81.5	
$[\alpha]_D^{20}$ (1% acetone), *degrees* ...	+84.5	+85	+84.8	+83.9	+79.6	+84.4		+83.7	+79.9	+84.2			+79.9	
Vitamin D_3-cholesterol														
M.p., °C....	118–119		117–118		114–116	116–117		112–113			117–118			110–113
$[\alpha]_D^{20}$ (1% acetone), *degrees* ...	+25.5		+22.8		+19	+25.1		+18.4			+24.6			+17.8

preparation of standard purity. Pure crystalline vitamin D_3 itself is difficult to obtain, the yields varying from 40 to 80 per cent.

It is a wide-spread assumption that pure crystalline vitamins D_2 and D_3 are stable compounds. From our experience, we cannot concur entirely in this opinion on the basis of observed physicochemical characteristics. Such stability data under various conditions are illustrated by Table III.

DISCUSSION

The various nitrobenzoic esters of vitamins D_2 and D_3 can be stored for at least 5 years at room temperature without decomposition. They can be rigidly purified by recrystallization until their physicochemical data conform to the characteristics in Table I. Pure crystalline vitamins D_2 and D_3 with constants conforming to those in Table II can be obtained by direct saponification with methanolic KOH from these esters.

Pure crystalline vitamin D_2, when sealed in amber evacuated ampules and stored in the refrigerator, does not change in appearance and in its physicochemical constants up to 9 months. With storage at refrigerator temperatures under air, decomposition occurs after 1 to 2 months, as indicated by the appearance of small oil deposits in the crystalline mass, together with a decrease of the melting point and of the rotation. A similar detectable decomposition is noted after storage in sealed, air-filled ampules at room temperature for 2 to 3 days. Inert gases such as nitrogen or carbon dioxide very materially diminish, but do not prevent, the decomposition.

Deterioration of pure crystalline vitamin D_3 is negligible after 12 months storage in amber evacuated ampules at refrigerator temperatures. In general it is more stable than vitamin D_2 when tested in the same manner. It seems well suited to serve as a standard reference preparation, provided it is stored under proper conditions. It also seems advisable to prepare fresh material every 9 to 12 months from rigidly purified esters with a standardized saponification procedure.

Vitamin D_3-cholesterol, although suited for the economical production of a crystalline vitamin D_3 preparation, shows a slightly inferior stability, as compared with vitamin D_3.

The above results indicate strongly that crystalline vitamin D preparations, unless handled with great care, are apt to lose potency in short order. Crystalline vitamin D preparations should be stored under optimal conditions and if the compounds are to be used for chemical studies their physicochemical constants should be rechecked if aged more than 12 months, although such changes on the basis of acceptable standards (40,000 international antirachitic units per mg.) are not demonstrable by biological tests on rats or chickens.

Stability of Vitamin D on Basis of Biological Assay

The degree of utilization of vitamin D differs materially, depending on the medium in which it is administered. Lewis (10) demonstrated that crystalline vitamin D_2 is clinically more effective when administered in the daily ration of milk to the child than when given in oily solution. His findings in this regard have been confirmed by Eliot *et al.* (11) and by Shelling (12).

Reports on the stability of vitamin D in various menstrua, however, are thus far meager and have not definitely settled all aspects of the question. Bourdillon, Bruce, and Webster (13) studied the stability of olive oil solutions of irradiated ergosterol. They found that such solutions, when stoppered and kept at 0°, showed no deterioration, but that the vitamin D values of similar samples kept at room temperature deteriorated materially. The authors estimated, by extrapolation of their data, that the solutions kept at room temperature would lose half of their potency in 3 years.

Bruce, Kassner, and Phillips (14) studied this problem in a more detailed manner. Solutions of crystalline vitamin D_2 in olive oil, cod liver oil, halibut liver oil, and paraffin oil were examined for stability; all solutions were sealed under nitrogen and were kept at 0°. These studies indicated that deterioration of the vitamin D occurred in all of the menstrua with the exception of the solutions in paraffin oil. However, the changes noted were not great, and the authors felt that the losses were within the limits of the experimental error inherent in biological assays for vitamin D. Such was certainly the case where one solution showed an apparent deterioration of 35 per cent after 11 months storage, but only 7 per cent after 19 months. Natural vitamin D kept at 0° under nitrogen for periods up to 3 years showed no change, except that the solutions in halibut liver oil seemed to be consistently less potent after various periods of storage.

On the other hand, Shelling (15) has shown that aqueous emulsions of oily solutions of vitamin D, although clinically very effective, deteriorate rapidly in the presence of air. Under these conditions, the antirachitic values of such emulsions deteriorated more than 90 per cent in 6 months. However, when the emulsions were stored for corresponding periods under nitrogen, only minor losses were observed. Studies on the stability of vitamin D_2 in water and milk were also reported by Supplee *et al.* (16). These authors reported that propylene glycol solutions of vitamin D were unstable when diluted with water, but entirely stable when diluted with milk during the period of assay.

According to Fuchs and van Niekirk (17), crystalline vitamin D_2 sealed in ampules under air loses from 35 to 55 per cent of its potency during 2 years storage in the dark. The literature, therefore, indicates that the

stability of vitamin D_2 to aging, either as crystals or in solutions, is dependent largely on the protection of the vitamin from oxygen.

We have been interested for some time in the stability of vitamins D_2 and D_3 in various menstrua. This paper presents a comparison of the relative stability of suspensions of vitamin D in water and milk and of solutions in vegetable oils or in propylene glycol. In view of the reports in the literature, it has seemed desirable to report stability data on solutions kept under the most severe conditions of storage likely to be encountered in practice.

TABLE IV

Recalcification Values

Product	Daily dosage level (10 days)			
	2.5 mg.	7.5 mg.	20 mg.	
U.S.P. reference cod liver oil, No. 2-36. Distribution date, Oct. 17, 1936; dilutions with peanut oil; dose in 0.1 cc..	1.21+	2.85+	3.17+	
	0.00625 γ	0.01875 γ	0.05 γ	0.1 γ
Drisdol, Lot DP-3319, No. 917. Room temperature 3 yrs.; dilutions with propylene glycol; dose in 0.1 cc. (Sample A)	1.37+	2.79+	3.38+	Not tested
Lot DP-3319, No. 917. Room temperature 3 yrs.; stock solution in water, then added to milk; dose in 4 cc. by stomach tube (Sample B).. ...	1.3+	3.0+	3.25+	3.44+
Lot DP-3319, No. 917. Same stock (water) solution as used for milk, diluted with distilled water; dose in 4 cc. by stomach tube (Sample C)	0.11+	0.36+	0.88+	1.55+

Methods

The several preparations were tested for vitamin D according to the procedures described in the United States Pharmacopoeia XI, or Volume XI, Second supplement. Eight to twelve animals were employed at each dosage level tested. All tests were completed prior to March, 1941.

Stability of Crystalline Vitamin D_2 in Various Solvents

Table IV presents a comparison of the antirachitic potency of the U.S.P. reference oil and of crystalline vitamin D_2 in propylene glycol, when

diluted (1) with propylene glycol, (2) with water, and (3) with milk. These data were obtained in 1936, at which time the solution of vitamin D_2 in propylene glycol (drisdol, Lot DP-3319) stored at room temperature under test was more than 3 years old. It had not changed in potency during that time.

Water—Vitamin D_2 in propylene glycol, when diluted with water under the conditions described by Supplee *et al.*, deteriorated markedly; *i.e.*, a loss of more than 75 per cent occurred during the period of the bioassay even though the samples of aqueous supplement were stored under refrigerator conditions. When the above freshly prepared aqueous stock

TABLE V

Effect of Storage at 40° on Potency of Solutions of Vitamin D_2 in Propylene Glycol

Length of storage	No of lots tested	Average recalcification values (+)									
		½ unit		¾ unit		1 unit		1½ units		2 units	
		R.O.*	Vitamin D†	R O	Vitamin D	R.O.	Vitamin D	R.O.	Vitamin D	R.O.	Vitamin D
wks											
Fresh	168‡	1.16	1.60	2.49	2.87	2.76	3.06	2.99	3.54	3.32	3.44
3	42	1.28	1.84	2.51	3.10	2.87	3.16	—	—	3.47	3.66
13	36	1.25	1.52	2.47	2.89	—	—	—	3.46	3.31	3.51
17	6	—	—	2.56	2.61	—	—	—	3.36	3.44	—
26	50	0.95	1.49	2.25	2.83	—	—	—	3.23	3.16	3.13
30	4	—	—	2.54	2.81	—	—	—	3.39	3.43	—
52	50	1.15	1.57	2.26	2.78	2.79	3.12	3.10	3.32	3.21	3.38
114	25	—	—	—	—	2.70	3.10	3.00	—	—	—
169	2	—	—	—	—	2.70	3.07	3.00	—	—	—
229	2	—	—	—	—	2.70	3.05	3.00	—	—	—

* U.S.P. reference oil.

† Vitamin D preparation. The estimated potencies are based on the assumption that 1 mg. contains 40,000 U.S.P. units.

‡ Most lots were tested at three dose levels. The figures given are averages of all available data on these 168 lots.

solution of drisdol was further diluted so that the supplement samples were suspended in milk, vitamin D assay values of the reference oil, drisdol Lot DP-3319 in propylene glycol (Sample A), or dilutions of Sample A in milk differed in no essential respect. Our observations indicate that drisdol *per se* is stable, but confirm both those of Supplee *et al.* and those of Shelling as to the instability of vitamin D in water (watery dilutions of propylene glycol solutions) and those of the former authors when the vitamin D preparation was diluted with milk.

Propylene Glycol—Solutions of crystalline vitamins D_2 and D_3 were prepared to contain 0.0257 per cent of the vitamin by weight. Samples were

stored in full amber bottles at 5°, at room temperature, and at 38–40°. The bottles were well sealed, but no attempt was made to displace air with CO_2 before they were sealed. The incubator samples were subsequently retested at somewhat longer intervals. Refrigerator samples were assayed occasionally simply as a control procedure. The results of these studies are shown in Tables V and VI.

Vegetable Oils—Solutions of crystalline vitamins D_2 and D_3, 0.0257 per cent in sesame and corn oils, were prepared and stored under the same conditions as described above. The incubated samples were kept in one

TABLE VI

Effect of Storage at 40° on Potency of Solutions of Vitamin D_3 in Propylene Glycol*

Length of storage	Average recalcification values (+)							
	½ unit		¼ unit		1 unit		2 units	
	R O.†	Vitamin D‡	R. O.	Vitamin D	R. O.	Vitamin D	R. O.	Vitamin D
wks.								
0	1.10	1.20	2.25	2.38	—	—	3.12	3.39
8	0.75	1.57	1.86	2.71	—	—	3.00	3.28
13	0.94	1.63	1.94	2.75	—	—	3.00	3.30
17	1.55	1.50	2 10	2.55	—	—	2.55	3.15
22	1.22	1.60	—	—	2.80	3.00	3.50	3.50
52	—	—	—	—	2.64	2.70	—	—
67	—	—	—	—	2.71	2.88	—	—
156	—	—	—	—	2.53	2.83	—	—

* Tests of material by physicochemical and biological methods indicated that it contained 85 to 90 per cent of pure crystalline D_3.

† U.S.P. reference oil.

‡ Vitamin D preparation. The estimated potencies are based on the assumption that 1 mg. contains 40,000 U.S.P. units.

case up to 31 months; in other cases only those at room temperature received extensive study. The results are shown in Table VII.

A comparison of assay data of Tables VI and VII indicates that oily solutions are antirachitically less potent in comparison with solutions in propylene glycol. It is suggested that either the rancidity of the oil or incomplete absorption may explain these differences.

Milk—Solutions of vitamin D_2 in propylene glycol were diluted with bulked samples of Grade A milk so that each quart bottle on refilling contained 400 to 2500 units (1.75 to 10 drops of drisdol). The fortified samples from a single delivery from a large distributor were stored in the refrigerator for periods of time over which the milk would be entirely satisfactory for human consumption; *i.e.*, up to 8 days. An unopened 8 day-old bottle was

used to prepare the oral supplements for each day of the assay. After careful correction of all assays, because of the antirachitic value of the milk

TABLE VII

Effect of Storage under Various Conditions on Stability of Oily Solutions of Vitamins D_2 and D_3

Solvent	Vitamin	Length of storage	Temperature	Average recalcification values (+)	
				R. O.* (1 unit)	Vitamin D† (1 unit)
		mos.	°C.		
Corn oil	D_2	0		2.61	2.89
" "	"	31	40	2.74	2.83
Sesame oil	"	39	Room	2.74	2.83
" "	"	54	"	2.68	2.77
				½ unit	½ unit
Corn oil	D_3	0		2.62	2.90
" "	"	12	40	2.62	2.69
" "	"	15	40	2.71	2.88
" "	"	31	40	2.84	2.93

* U.S.P. reference oil.

† Vitamin D preparation. The estimated potencies are based on the assumption that 1 mg. contains 40,000 U.S.P. units.

TABLE VIII

Effect of Storage under Various Conditions on Potency of Ertron

Lot	Duration of storage	Temperature	Average recalcification values (+)					
			½ unit		1 unit		1½ units	
			U.S.P. reference oil	Ertron	U.S.P. reference oil	Ertron	U S P. reference oil	Ertron
	wks.	°C.						
A	0		—	1.52	2.59	2.67	—	—
B	0		—	—	2.50	2.65	—	—
A	4	5	—	—	2.55	2.15	3.00	2.75
B	8	Room	—	—	2.75	2.38	—	—
"	11	"	—	—	2.70	2.22	—	—
"	6	38	—	—	2.75	2.25	—	—
A	13	38	—	—	2.75	2.00	—	2.50

per se, no evidence of deterioration of the vitamin D potency of the fortified milk was demonstrable.

A test of the stability of vitamin D_2 in condensed milk was also made.

Samples were fortified in the following manner. 14.5 ounce cans of a well known brand of condensed milk were purchased on the open market. An area on the surface of the cans was sterilized with an antiseptic and punctured. 0.1 cc. of a propylene glycol solution of vitamin D_2 in suitable concentration was then added to the contents of each can in order to bring the antirachitic value up to 500 U.S.P. units per can. All cans were then resealed with solder (air not replaced), and sterilized at 120° for 30 minutes. The samples were stored under various temperature conditions as previously outlined. The incubator samples (38.0-40.0°) were assayed after 6 months storage, and the samples at room temperature at intervals up to 15 months. None showed any loss in potency.

TABLE IX

Vitamin D Assays of Additional Samples of Ertron and Vitamins D_2 and D_3

U.S.P. reference oil		Ertron, No. 1109, received June 14, 1940, stored in ice box		Ertron, No 997, received Feb. 21, 1940				Vitamin D_2 in oil		Vitamin D_3 in oil	
				Incubated at 38° 11 mos		Stored in ice box 11 mos.		1 million units vitamin D per cc., prepared Dec. 17, 1938; stored in ice box			
Dose	Plus values	Plus values	Per cent of I U reference oil	Plus values	Per cent of I U. reference oil	Plus values	Per cent of I.U. reference oil	Plus values	Per cent of I U. reference oil	Plus values	Per cent of I.U. reference oil
units											
½	2.06										
1	2.61	2.57	95.0	1.75	37.5	2.66	105.0	2 97	130.0	3.00	132.5
1½	2.97	3.03									
% of claim present			99.15		37.5		105.0		130.0		132.5

Stability of Ertron

Ertron is a form of activated ergosterol prepared by the Whittier process.[1] An ethereal solution of the activated material is dried on casein to obtain the commercial product. Stability tests by biological assays were conducted on the capsulated material as purchased. On the basis of earlier ultraviolet absorption experiments the activity of this preparation diminished 50 per cent or more after storage for 3 months at room temperature. The observed antirachitic effects of ertron with time are illustrated in Tables VIII and IX.

The antirachitic potency of different lots of ertron purchased through usual channels varied over a range of 75 to 105 per cent of that claimed (50,000 units per capsule).

[1] Manufactured by the Nutrition Research Laboratories, Chicago.

DISCUSSION

There is nothing to suggest that either vitamin D_2 or D_3 in propylene glycol shows any change in potency when stored in amber bottles at incubator temperatures (38.0°) even though sealed in the presence of air. In the case of vitamin D_2, the tests cover samples aged 53 months; in the case of vitamin D_3, they cover samples aged 36 months. Since these conditions are as drastic as would ever conceivably be imposed on such pharmaceutical products, it may be stated that for all practical purposes the solutions are stable. The solutions of these vitamins in corn oil also show no loss in potency during 30 months in the incubator.

On the other hand, ertron, which was satisfactory as to potency relative to claims when fresh, frequently shows some deterioration after only 4 weeks in the refrigerator. During 3 months in the incubator it loses more than one-third of its potency.

Solutions of vitamin D_2 in propylene glycol do not lose their potency when dissolved either in bottled whole milk kept up to 8 days in the refrigerator, or in canned condensed milk stored for 6 months at 40° or for periods up to 15 months at room temperature.

It is also apparent from these data that both vitamins D_2 and D_3 in the crystalline form are more potent than the 40,000 I.U. or U.S.P. units per mg. as generally recognized and reviewed by Remp and Marshall (18). In general, the reference oil uniformly produces a lesser degree of recalcification than does the corresponding dose of these vitamins calculated on the 40,000 units per mg. basis in the rat and in the case of vitamin D_3 in both rats and chickens. As calculated from more than 400 separate satisfactory assays, it appears that these crystalline vitamins are undervalued in the literature by at least 10 and more probably by 15 per cent.

Every comparative test of the two vitamins shows that they behave exactly alike as to potency in the rat, and that whatever potency figure is set for the one must be accepted for both. However, preparations of vitamin D_3 are more invariable in their biological characteristics. This vitamin should serve admirably as a standard reference preparation, either as a crystalline product of uniform chemical and physical characteristics, or more conveniently as an oil or propylene glycol solution. Such a preparation would serve equally well for rat and chicken assays.

Chemical tests of activated ergosterol or 7-dehydrocholesterol indicated that the characteristics of these products when impure, *i.e.* mixed with antirachitically inactive products, were materially less stable than the crystalline products with a constant melting point and optical rotation.

SUMMARY

Physicochemical studies of the various nitrobenzoic esters of vitamins D_2 and D_3 indicate that no decomposition is demonstrable after storage at

room temperatures for at least 5 years. The stability of crystalline vitamin D_2 or D_3 in the presence of air, nitrogen, or CO_2 is materially less than when stored in an evacuated ampule. Vitamin D_2, when stored in evacuated amber ampules under refrigerator temperatures, undergoes no change in its physicochemical characteristics over a storage period up to 9 months. Vitamin D_3 in general is somewhat more stable than vitamin D_2 and no definite change is demonstrable after storage under optimal conditions for a period of 12 months.

Solutions of vitamins D_2 or D_3 in propylene glycol or vegetable oils retain their potency over long periods of time at a temperature of 40°. When such solutions are dissolved in milk, the vitamin D potency of the fortified milk remains unchanged over periods of time corresponding to the usefulness of the menstruum as a food.

The data presented indicate that the usually accepted antirachitic value of 40,000 U.S.P. units per mg. for calciferol or crystalline vitamin D_2 and crystalline vitamin D_3 is 10 to 15 per cent low.

BIBLIOGRAPHY

1. Fritz, J. C., Halpin, J. L., Hooper, J. H., and Kramke, E. H., *Ind. and Eng. Chem.*, **34**, 979 (1942).
2. Baird, F. D., Ringrose, A. T., and MacMillan, M. J., *Poultry Sc.*, **18**, 35 (1939).
3. Jung, A., *Cong. internat. chim. tech. ind. agric., Compt. rend. VI cong.*, Budapest, **2**, 449 (1939).
4. Windaus, A., Deppe, M., and Wunderlich, W., *Ann. Chem.*, **533**, 118 (1937).
5. Askew, F. A., Bourdillon, R. B., Bruce, H. M., Callow, R. K., Philpot, St. L. F., and Webster, T. A., *Proc. Roy. Soc. London, Series B*, **109**, 488 (1932).
6. Windaus, A., von Werder, F., and Gschaider, B., *Ber. chem. Ges.*, **65 B**, 1006 (1932).
7. Anderson, F. W., Bacharach, A. L., and Smith, E. L., *Analyst*, **62**, 430 (1937).
8. Schenk, F., *Naturwissenschaften*, **25**, 159 (1937).
9. U. S. patent 2,264,320 (1941).
10. Lewis, J. M., *J. Pediat.*, **6**, 362 (1935); **9**, 308 (1936).
11. Eliot, M. M., Nelson, E. M., Barnes, D. J., Browne, F. A., and Jenss, R. M., *J. Pediat.*, **9**, 355 (1936).
12. Shelling, D. H., *J. Pediat.*, **10**, 748 (1937).
13. Bourdillon, B. T., Bruce, H. M., and Webster, T. A., *Biochem. J.*, **26**, 522 (1932).
14. Bruce, H. M., Kassner, E. W., and Phillips, G. E., *Quart. J. Pharm. and Pharmacol.*, **11**, 46 (1938).
15. Shelling, D. H., *Proc. Soc. Exp. Biol. and Med.*, **35**, 660 (1937).
16. Supplee, G. C., Ansbacher, S., Bender, R. C., and Flanigan, G. E., *J. Biol. Chem.*, **114**, 95 (1936).
17. Fuchs, L., and van Niekirk, J., *Biochem. Z.*, **277**, 32 (1935).
18. Remp, D. G., and Marshall, I. H., *J. Nutrition*, **15**, 527 (1938).

STUDIES ON THE METABOLISM OF ZINC WITH THE AID OF ITS RADIOACTIVE ISOTOPE

II. THE DISTRIBUTION OF ADMINISTERED RADIOACTIVE ZINC IN THE TISSUES OF MICE AND DOGS

By G. E. SHELINE, I. L. CHAIKOFF, H. B. JONES, AND M. LAURENCE MONTGOMERY

(From the Division of Physiology, University of California Medical School, Berkeley, and the Division of Surgery, University of California Medical School, San Francisco)

(Received for publication, April 26, 1943)

The value of Zn^{65} as a tracer element in the study of the metabolism of zinc was pointed out in Paper I (1). The data recorded here deal with the turnover (uptake and loss) of intravenously injected labeled zinc in the tissues of mice and dogs.

EXPERIMENTAL

The preparation of labeled zinc containing Zn^{65}, as well as the method for its measurement, has already been described (1).

Animals Used; Dogs—Five dogs were injected intravenously with labeled zinc prepared as the chloride. At various intervals thereafter the animals were anesthetized by means of an intraperitoneal injection of nembutal. A cannula was introduced into the carotid artery immediately and the animal bled. Whole organs were then removed. In the case of bone, a single femur was taken from each dog. Samples of skeletal muscle were removed from the thigh. Samples of skin plus hair were obtained from the backs of the animals.

The whole organs or tissues were dried in an electric oven maintained at 110°. The dried material was then ashed in an electric muffle thermostatically controlled at 450°, for 12 to 18 hours. The ash was extracted with hot concentrated hydrochloric acid, filtered, diluted to volume, and the Zn^{65} determined. 18 hours were insufficient for complete ashing of the larger amounts of tissue; in these cases the residue was extracted with hot concentrated HCl, filtered, and the insoluble portion reashed at the same temperature. After two or three ignitions, ashing was usually complete. On occasion, however, small amounts of black carbonaceous material remained; this was treated with HCl and the residue filtered out. The radioactivity of the residue was determined separately. The filtrates obtained from repeated ashing were combined and diluted to volume. Determination of the Zn^{65} content was made after precipitation of the zinc as the carbonate (1).

The distribution of labeled zinc in blood corpuscles and plasma was determined in three large dogs. Immediately after withdrawal of blood from the external jugular vein, the sample was heparinized and 1 cc. portions transferred to rectangular dishes, 2 × 6 cm., made from aluminum-foil that had been lined with lens paper. The remaining blood was centrifuged for 5 minutes at 3500 R.P.M. and the plasma decanted from the cells. 1 cc. samples of both plasma and cells were transferred to small aluminum plates. The remaining cells were shaken with approximately twice their volume of freshly prepared Ringer's solution, the pH of which had been previously adjusted to 7.4. These cells were again centrifuged and the supernatant layer poured off. 1 cc. samples of the washed cells were analyzed for labeled zinc.

Mice—Two series of animals were used. In the first group there were twenty-one mice; three or four were sacrificed at each of six time intervals, ranging from 45 minutes to 170 hours after intravenous injection of the labeled zinc. In the second group three mice were killed at each of the six time intervals.

The mice were sacrificed by rapid decapitation with a pair of surgical scissors. The first drop or two of blood from the severed carotid artery was discarded, and the next few drops were collected on a clean glass plate. This blood was drawn into medicine droppers and approximately 0.1 gm. samples weighed out. Since pancreatic tissue in the mouse can be more readily seen macroscopically when the circulatory system of the gut is intact, a mouse with intact circulation was anesthetized and the pancreas exposed. With this preparation as a guide, the pancreatic samples were taken from the decapitated animals. Included with the pancreatic tissue were the mesentery of the loop of the duodenum, the base of the mesentery, and the portion of tissue immediately adjacent to the pylorus. The tissues were weighed and uniformly spread over a rectangular piece of blotting paper measuring 2 × 6 cm. and wrapped in cellophane. The samples were allowed to dry before their radioactivity was determined. With the exception of liver, skeletal muscle, skin, and bone, of which only portions were taken for analyses, the activity of each *whole* organ was determined.

Results

Plasma and Red Blood Cells—In each of three dogs the contents of Zn^{65} in plasma, red blood cells, and whole blood were measured at intervals of from 3 minutes to 48 hours after the injection of labeled zinc. A typical result is shown in Fig. 1. In the five dogs recorded in Table I, a single blood sample was obtained when each animal was sacrificed and the Zn^{65} content per cc. of whole blood determined. The deposition of Zn^{65} in whole blood of the mouse at various intervals up to 170 hours is shown in Fig. 2.

Radiozinc is rapidly removed from the *plasma* of the dog (Fig. 1). At the earliest interval, namely 3 minutes after the introduction of the labeled zinc into the blood stream, each cc. of plasma contained 0.025 per cent of the injected Zn^{65}. 30 minutes after the injection each cc. of plasma contained less than 0.002 per cent, and in 10 hours the injected radiozinc had practically disappeared from the plasma.

The Zn^{65} content per cc. of *red blood cells* at the 48 hour interval was 0.0017 per cent in Dog III (Fig. 1), 0.0035 per cent in Dog I, and 0.004 per cent in Dog II. These values represent increases above the amounts found

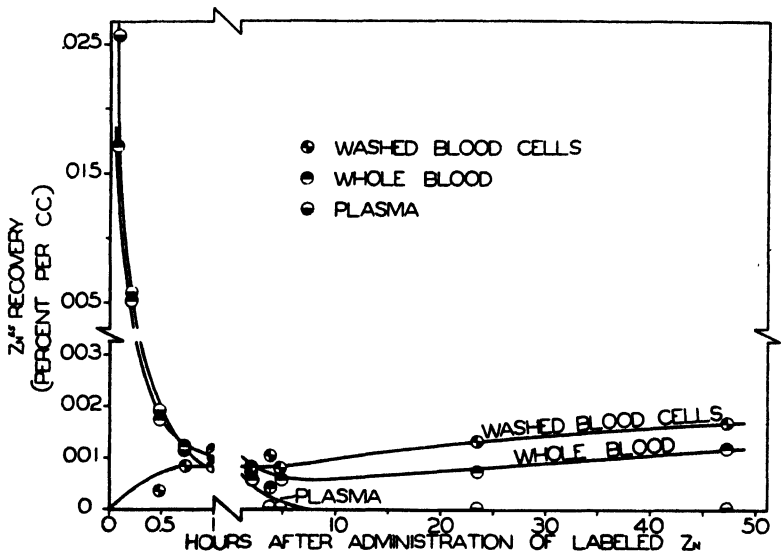


FIG. 1. The Zn^{65} content of plasma, red blood cells, and whole blood of Dog III. This dog weighed 17.0 kilos and received intravenously 16.3 γ of labeled zinc.

at the 1st hour. Thus each cc. of the washed red blood corpuscles of Dog I contained 0.0014 per cent of the injected Zn^{65} at the 50 minute interval; the cells of Dog II contained 0.0026 per cent per cc. at the 52 minute interval; the cells of Dog III contained 0.0008 per cent per cc. at the 59 minute interval. No significant differences in the Zn^{65} content of washed and unwashed cells were noted at the later intervals. It is probable that the Zn^{65} content shown for cells at the very early intervals is higher than their true content, since at these times the cells were separated from plasma that contained more than 25 times as much Zn^{65} as did the corpuscles. At the late intervals practically all of the radiozinc found in blood was present in the corpuscles.

In the five dogs recorded in Table I, the total amount of Zn^{65} contained in the blood stream (3 to 170 hours after the injection of the labeled zinc) represented from 2 to 4 per cent of the administered Zn^{65}. These values

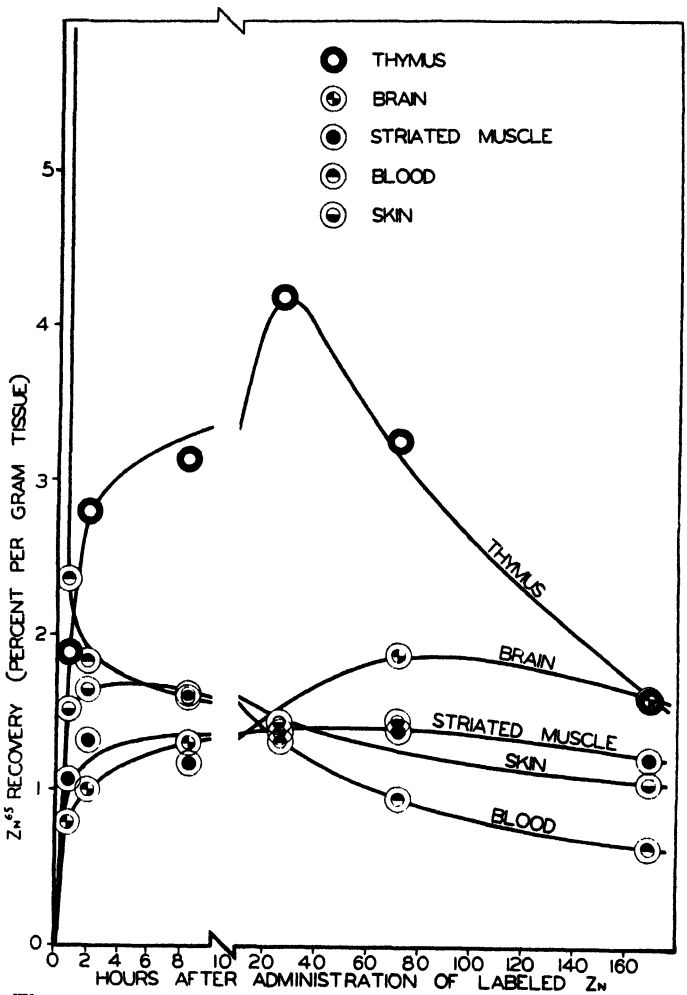


FIG. 2. The recovery of intravenously injected labeled zinc in thymus, brain, striated muscle, whole blood, and skin of the mouse. Each value is the average of three to seven separate determinations on as many animals. The mice weighed 18 to 23 gm. and each received 0.33 to 1.6 γ of labeled zinc.

were calculated on the assumption that blood constitutes 9 per cent of the body weight.

Liver, Pancreas, and Kidney—The highest concentrations of Zn^{65} were found in these tissues (Table I and Fig. 3). At the earliest intervals,

namely ¾ hour and 2 hours after the injection of labeled zinc, 19 to 43 per cent of the administered Zn^{65} was found per gm. in each of these three tissues of the *mouse*. A rapid turnover of zinc in these three tissues is suggested by the rates at which the tissues acquire as well as lose Zn^{65}. At the 26 hour interval, the Zn^{65} content of the liver and kidney of the *mouse* was reduced to about one-half of that found at the 2 hour interval, whereas at 170 hours the liver, kidney, and pancreas of the *mouse* contained 1.8 to 4.0 per cent per gm. of the injected Zn^{65}.

Maximum deposition in these tissues of the *dog* was observed at the 8 hour interval (Table I). At this time the liver of the *dog* contained 0.34 per cent of the injected Zn^{65} per gm., the kidney 0.21 per cent per gm., and the pancreas 0.28 per cent. The rate at which Zn^{65} was lost by these tissues in the *dog*, however, was much slower than in the mouse.

The whole liver, on account of its size, contained the largest fraction of the injected Zn^{65} at the early intervals (Tables I and II). In the *dog*, the whole liver held 38 per cent of the administered Zn^{65} at the 3 hour interval, and as late as 24 hours this organ still contained 33 per cent. The whole liver of the *mouse* contained 25 per cent of the injected Zn^{65} at 2 hours and 11 per cent at 26 hours. At the late intervals (94 and 170 hours) the Zn^{65} content of the dog's whole liver is exceeded only by the amount present in total striated musculature (Table I).

Gastrointestinal Tract—The stomach, small intestine, and large intestine of the *mouse* showed a rapid deposition of the injected zinc (Fig. 4 and Table II). At 2 hours, 10 per cent of the administered Zn^{65} was deposited in each gm. of stomach, 13 per cent in each gm. of small intestine, and 9 per cent per gm. of large intestine. The turnover of Zn^{65} in these tissues is also rapid, for at 72 hours their content of the radioisotope of zinc was reduced to 5.4 to 5.9 per cent per gm.

A more extensive study was made in the *dog*. The deposition of Zn^{65} was determined in the stomach, the cecum plus colon, and in various divisions of the small intestine. In four of the five dogs separate determinations were made for mucosa and muscular layer of the small intestine.

At the times of examination the *dog's* stomach contained 0.029 to 0.043 per cent of the injected dose per gm. (Table I). 0.032 to 0.084 per cent per gm. was found in the muscular part of the small intestine. Little or no difference was observed in the Zn^{65} content per gm. of the various quarters of the small intestine, but the largest total amount of Zn^{65} was found in the first quarter. Appreciable amounts of the Zn^{65} were also found in the mucosal layer of the small intestine. The cecum plus large intestine contained from 0.036 to 0.076 per cent per gm. at the various intervals.

The entire gastrointestinal tract of the *dog* contained 13 per cent of the

TABLE I

Distribution of Zn^{65} in Tissues of Dog after Intravenous Injection of Labeled Zinc

Dog No.	XI		X		XIII		XIV		XII	
Weight, *kg.*	4.7		4.0		3.5		3.8		5.2	
Interval after injection of labeled Zn, *hrs.*	3		8		24		94		170	
Labeled Zn injected, *mg*	0.00815		0.0130		0.00652		0.00815		0.00815	
Tissue	Per cent recovery per whole organ (*a*), per 100 gm. tissue (*b*)									
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Whole blood*	3.2	0.76	2.7	0.75	1.9	0.61	4.2	1.2	4.1	0.88
Pancreas	2.3	24	3.1	28	1.2	17	2.0	24	0.69	6.8
Liver	38	32	34	34	33	30	11	12	3.5	2.5
Kidneys (2)	3.2	16	3.9	21	2.5	14	1.3	7.3	0.90	3.6
Stomach	0.88	2.9	1.3	3.7	0.93	3.2	1.2	4.3	0.81	2.7
Small intestine										
Muscle layer										
1st†	2.3	8.4	3.2‡	11‡	1.9	7.1	1.3	6.4	0.82	3.2
2nd	1.2	7.3	3.0‡	12‡	1.0	8.0	1.0	6.8	0.79	3.3
3rd	0.96	7.9	2.6‡	13‡	0.86	7.9	0.84	6 4	0.56	3.2
4th	0.81	6.4	1.3‡	8.1‡	0.63	6.9	0.98	5.6	0.59	3.8
Average		7.5		11‡		7.5		6.3		3.4
Total	5.2		10		4.5		4.1		2.8	
Intestinal mucosa	7.2				2.8		2.1		1.1	
Colon + cecum	0.81	3.6	1.1	6.9	1.2	6.6	1.4	7.6	1.0	4.4
Skeletal muscle§	9.5	0.51	9.6	0.60	13	0.92	25	1.7	36	1.8
Diaphragm	0.088	0.80	0.18	1.3	0.12	1.4	0.22	2.2	0.13	0.86
Uterus			0.45	9.7	0.45	7.1	0.75	10		
Heart	0.58	2.0	1.1	4.6	0.70	3.1	1.1	4.8	0.98	2.8
Femur		1.2				3.2		2.2		2.0
Thyroids (2)	0.017	5.9	0.018	12	0.017	6.8	0.014	3.7	0.0052	1.7
Adrenals	0.027	6.4	0.054	10	0.038	6.1	0.025	3.9	0.014	2.5
Pituitary	0.12	7.3	0.0061	20	0.0021	6.9	0.0020	6.3	0.0020	5.7
Testes (2)	0.068	0.80							0.094	1.7
Ovaries (2)			0.038	7.0	0.020	5.6	0.022	3.4		
Brain	0.067	0.10	0.36	0.63	0.38	0.63	0.68	1.5	0.62	1.1
Cord	0.017	0.17	0.028	0.34	0.031	0.37	0.044	0.69	0.053	0.63
Lungs	0.63	2.5	0.73	3.4	0.79	4.0	1.1	4.6	0.71	2.7
Spleen	0.37	6.5	0.93	10	0.72	6.8	0.60	6.8	0.31	3.8
Parotid glands (2)	0.17	3.9	0.24	4.5	0.13	4.8	0.11	3.5	0.075	1.7
Skin and hair		0.82		0.73		3.5		1.7		
Intestinal lymph nodes	0.07	4.3	0.076	5.5	0.061	5.3	0.048	4.2	0.064	2.3
Peripheral lymph nodes	0.24	2.9	0.27	5.3	0.54	9.2	0.20	5.7	0.32	2.5
Bile in bladder at time of sacrifice		0.04		0.07		0.11		0.0		0.03

TABLE I—*Concluded*

* Calculated on the basis that blood constitutes 9 per cent of the body weight.

† Quarters measured by length.

‡ The figures so designated represent Zn^{65} recoveries in combined muscular and mucosal layers.

§ Calculated on the basis that muscle constitutes 40 per cent of the body weight.

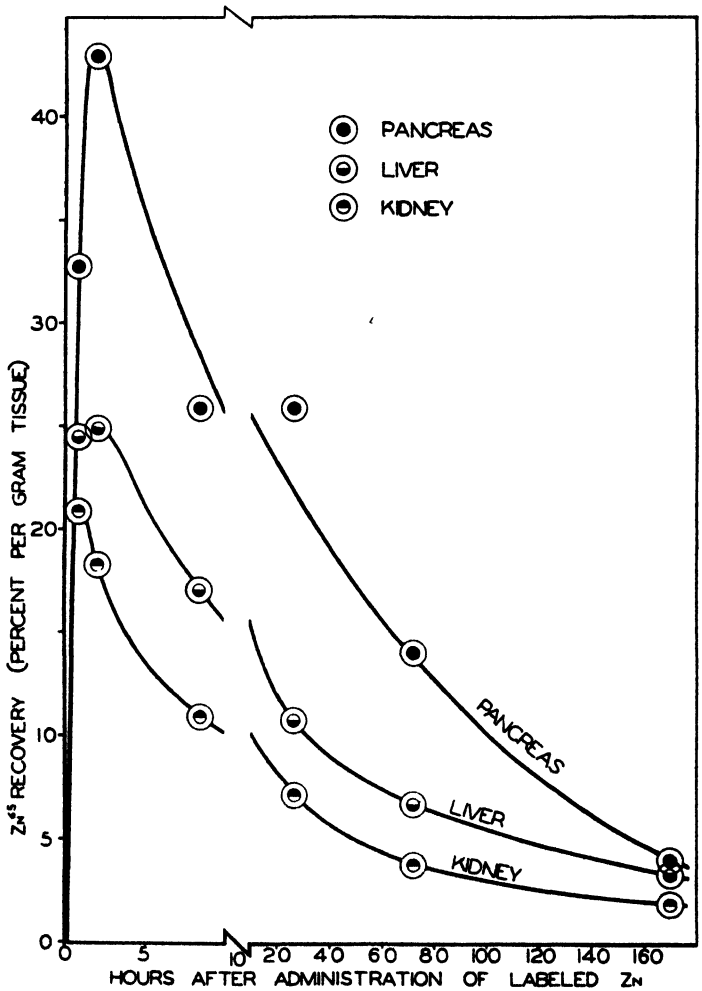


FIG. 3. The recovery of intravenously injected labeled zinc in pancreas, liver, and kidney of the mouse. Each value is the average of three to seven separate determinations on as many animals.

injected Zn^{65} at the 3 hour interval. At 24 hours this was reduced to 8 per cent, but at 170 hours it still contained 5 per cent. In the *mouse* the highest content for the whole gastrointestinal tract was observed at 8 hours

after the introduction of the radioisotope; at this time it contained 13 per cent of the injected Zn^{65}. At 170 hours it still contained over 2 per cent.

Muscle—In the *mouse* the content of Zn^{65} was determined in skeletal muscle (Fig. 2) and in heart tissue (Fig. 4 and Table II), whereas in the *dog* determinations were made in leg muscle, diaphragm, uterus, and heart (Table I). The zinc turnover of the skeletal muscle of the mouse is relatively slow. Although 1.1 per cent of the injected Zn^{65} was deposited per gm. of skeletal muscle as early as ¾ hour, the maximum deposited in this tissue was only 1.5 per cent per gm., and as late as 170 hours this tissue still contained 1.2 per cent per gm. Skeletal muscle in the dog also showed a

TABLE II

Per Cent of Intravenously Injected Zn^{65} Recovered per Whole Organ of Mouse

The mice weighed 17 to 23 gm. Each animal received intravenously from 0.33 to 1.6 γ of zinc as the chloride. Each value is the average of three to seven separate analyses on as many animals.

Tissue	Time after injection					
	¾ hr.	2 hrs.	8 hrs	26 hrs	72 hrs.	170 hrs
Pancreas	2.0	1.7	0.93	0.99	0.52	0.44
Liver	24	25	17	11	6.7	3.3
Kidneys	6.5	5.2	3.0	1.7	0.98	0.50
Stomach	0.83	1.1	1.0	0.99	0.59	0.37
Small intestine	4.5	5.8	8.4	3.8	2.2	0.96
Colon	1.6	2.4	3.8	2.0	1.1	0.83
Heart	0.38	0.54	0.44	0.48	0.29	0.14
Adrenals	0.040	0.040	0.026	0.048	0.021	0.011
Thymus	0.064	0.098	0.079	0.16	0.084	0.038
Brain	0.32	0.38	0.55	0.56	0.77	0.64
Lungs	0.98	1.1	0.97	0.72	0.46	0.26
Spleen	0.84	1.3	1.9	0.73	0.37	0.14
Lymph nodes	0.40	0.42	0.52	0.38	0.38	0.12

slow turnover of zinc. By 94 hours, 0.017 per cent was deposited per gm. of the dog's gastrocnemius muscle, and at the last interval, namely 170 hours, this muscle still contained about the same amount of Zn^{65}. Somewhat higher amounts were deposited in the dog's diaphragm: 0.022 per cent per gm. was found in this tissue at the 94 hour interval.

There is a more rapid turnover of zinc in cardiac than in skeletal muscle. Thus at the 2 hour interval, cardiac tissue of the mouse contained 6.5 per cent of the administered Zn^{65} per gm.; at the 170 hour interval it still contained 1.9 per cent per gm. The amounts of Zn^{65} deposited in the dog's heart per gm. were higher than in its gastrocnemius muscle at all intervals noted; at the 94 hour interval the dog's heart contained 0.048 per cent per gm., and at 170 hours 0.028 per cent per gm. was still present.

If it is assumed that the labeled zinc is uniformly distributed throughout the skeletal muscle and that this tissue represents approximately 40 per cent of the total body weight, then the total musculature of Dog XIV (94 hours)

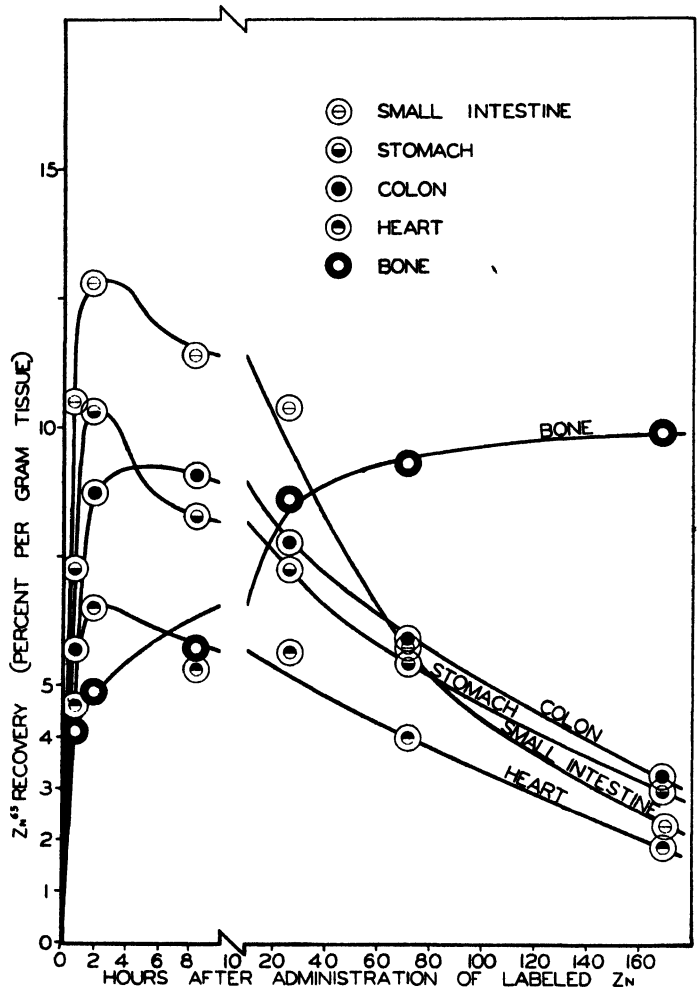


FIG. 4. Recovery of intravenously injected labeled zinc in small intestine, stomach, colon, heart, and bone of the mouse. Each value is the average of three to seven separate determinations on as many animals.

and Dog XII (170 hours) contained respectively 25 and 36 per cent of the administered Zn^{65}.

The Zn^{65} content of the uterus of three dogs was determined. Values of 0.07 to 0.10 per cent per gm. were found at 8, 24, and 94 hours.

Bone—Zn^{65} was rapidly deposited in the tibia of the mouse (Fig. 4), and this tissue continued to increase its content of radiozinc throughout the period of observation. Thus at 2 hours 5 per cent was deposited per gm. of

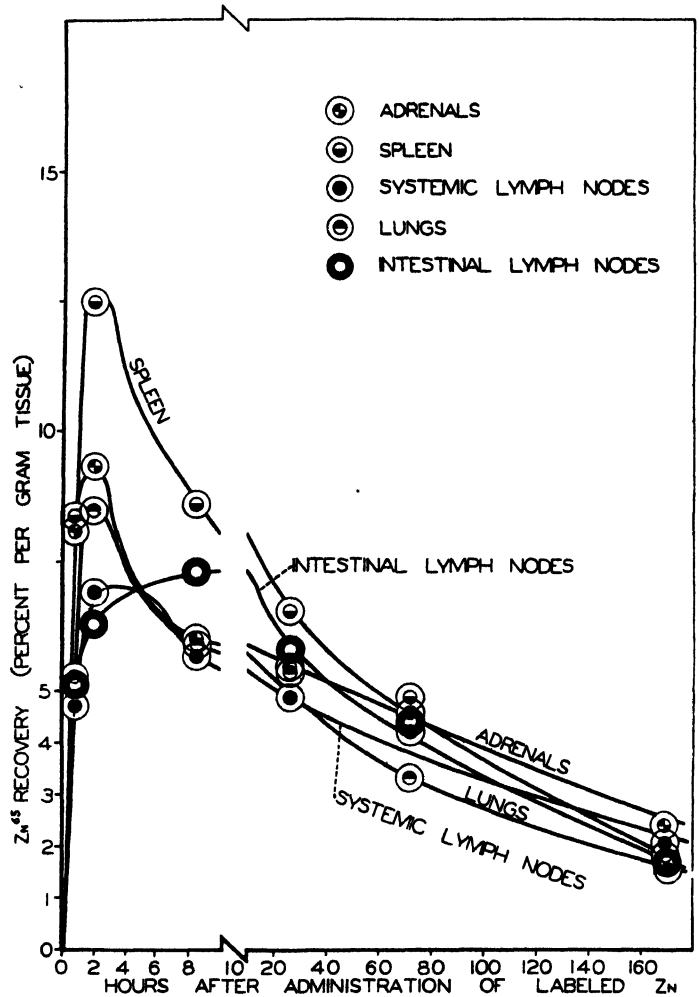


FIG. 5. The recovery of intravenously injected labeled zinc in adrenals, spleen, lymph nodes, and lungs of the mouse. Each value is the average of three to seven separate determinations on as many animals.

bone, and at the last time interval (170 hours) the radiozinc content of this tissue had doubled (9.9 per cent per gm.). The retention of radiozinc by the dog's femur is shown in Table I.

Endocrines—The pancreas has been dealt with above. In this section

will be considered the thyroid, adrenal, pituitary, and thymus. The most rapid turnover in these tissues was observed in the *dog's* pituitary (Table I). This gland showed an activity roughly similar to that of the kidney. The highest and lowest values were 0.20 and 0.057 per cent per gm.

The highest and lowest values for the Zn^{65} content of the thyroid gland of the *dog* were 0.12 and 0.017 per cent per gm. at the intervals examined (Table I).

In both dog and mouse the *whole* adrenal gland deposited from 0.01 to 0.05 per cent of the injected radiozinc (Tables I and II). Per gm. this tissue is quite active in the *mouse;* over 9 per cent of the injected Zn^{65} was found per gm. of adrenal gland at 2 hours (Fig. 5). The highest concentration of Zn^{65} in the dog's adrenal gland was observed at 8 hours; at this time it contained 0.10 per cent per gm. (Table I).

Nervous System—The turnover of zinc in the nervous system of the dog and mouse is slow when compared to that in such tissues as liver or kidney. As much as 0.015 per cent of the injected Zn^{65} was found per gm. of dog brain (Table I), and as much as 1.9 per cent per gm. of mouse brain (Fig. 2). Less than 1 per cent of the injected radiozinc was deposited in the entire brain of these animals (Tables I and II).

At the 3 hour interval the Zn^{65} content per gm. of dog liver was over 300 times that of its brain; at the 24 hour interval the ratio of Zn^{65} content in these tissues was 50:1; at the last interval, namely 170 hours, this ratio had decreased to 2:1.

The concentrations of radiozinc in liver and brain of the mouse were compared at 2, 26, and 170 hours. The ratios of Zn^{65} content per gm. of liver to gm. of brain found at these three intervals were 25, 8, and 2 respectively.

Spleen, Lymph Nodes, and Thymus—Spleen and lymph nodes in both mice (Fig. 5 and Table II) and dogs (Table I) showed an active zinc turnover; in the dog the whole spleen contained about 1 per cent of the injected Zn^{65} at the 8 hour interval; at this time the spleen of the mouse contained about 2 per cent.

The maximum deposition of Zn^{65} in the thymus of the mouse was about 4 per cent per gm. (Fig. 2). In contrast to most of the tissues, however, the thymus required 26 hours for this amount to be deposited.

DISCUSSION

In both dog and mouse the largest amount of the intravenously injected radiozinc appeared in the whole liver at the early intervals. The labeled Zn was not retained in this organ for long; at the 3 hour interval 38 per cent of the radiozinc was found in the dog's liver, whereas only 3.5 per cent was present in this organ at the 170 hour interval. The Zn^{65} that disappeared from the liver between these two intervals was not entirely lost in bile,

urine, and feces. It has been shown elsewhere (1) that in 7 days (168 hours) 17.5 to 21 per cent of the radiozinc injected intravenously into the dog appeared in the urine and feces; less than 0.5 per cent appeared in the bile in 7 days.[1] These observations suggest that the Zn^{65} lost by the liver between 3 and 170 hours is deposited in other tissues.

In contrast to tissues like liver, pancreas, kidney, and spleen (in which large amounts of radiozinc accumulate early, but much of it rapidly disappears) several tissues were characterized by either a slow deposition increasing with time or by maintenance of the small amounts deposited at the early intervals. These tissues were blood corpuscles, bone, nervous system, skin, and skeletal muscle.

Keilin and Mann's discovery that the enzyme carbonic anhydrase is a zinc-containing protein established for the first time the physiological importance of this metal in the animal organism (2). Their finding that carbonic anhydrase contains 0.31 to 0.34 per cent zinc and that the total zinc content of the red blood corpuscles can be accounted for by its presence in the enzyme has been confirmed by Hove *et al.* (3). Scott *et al.*, however, obtained more active preparations, the zinc contents of which were 0.15 to 0.2 per cent (4, 5). The wide-spread distribution of zinc in the animal body suggests that zinc is linked in animal tissues with compounds in addition to carbonic anhydrase. Thus zinc has been found in purified preparations of uricase (6, 7). The rôle of zinc in the storage of insulin in the pancreas has also been considered (8–10). The zinc content of the pancreas, however, is many times the amount necessary to account for its insulin content in the form of a zinc salt (9).

Despite the fact that the above evidence indicates that the physiological importance of zinc in the body lies in its association with organic molecules, probably protein, it would be premature at present to attempt to explain the distribution of radiozinc recorded in this investigation by the zinc-containing compounds identified so far.

SUMMARY

The distribution of *intravenously* injected zinc was measured in the tissues of the mouse and dog by means of its radioactive isotope (Zn^{65}). This procedure permitted the introduction into the blood stream of such small amounts as 0.33 to 1.6 γ in the mouse and 6.5 to 16.3 γ in the dog; these amounts did not alter significantly the zinc content of the animals.

1. Injected radiozinc rapidly disappears from the plasma. Measurable amounts of Zn^{65} were no longer detectable in the plasma of the dog 10 hours after its injection into the blood stream.

2. Radiozinc appeared early in the red blood cells. The Zn^{65} content in

[1] Unpublished observations.

these cells continued to increase during the entire period of observation (170 hours).

3. At the early intervals maximum deposition occurred in the liver. At the 3 hour interval the dog's liver contained 38 per cent of the injected radiozinc. At the 2 hour interval the liver of the mouse contained 25 per cent.

4. The most active turnover of radiozinc (uptake and loss) was observed in the liver, pancreas, kidney, and pituitary gland. The least activity was found in red blood cells, brain, skeletal muscle, and skin. The activities of spleen, gastrointestinal tract, adrenals, lungs, lymph nodes, bone, heart, and thymus were intermediate.

BIBLIOGRAPHY

1. Sheline, G. E., Chaikoff, I. L., Jones, H. B., and Montgomery, M. L., *J. Biol. Chem.*, **147**, 409 (1943).
2. Keilin, D., and Mann, T., *Nature*, **144**, 442 (1939); *Biochem. J.*, **34**, 1163 (1940).
3. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **136**, 425 (1940).
4. Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, **140**, 445 (1941).
5. Scott, D. A., and Fisher, A. M., *J. Biol. Chem.*, **144**, 371 (1942).
6. Davidson, J. N., *Biochem. J.*, **32**, 1386 (1938).
7. Holmberg, C. G., *Biochem. J.*, **33**, 1901 (1939).
8. Scott, D. A., and Fisher, A. M., *J. Clin. Inv.*, **17**, 725 (1938).
9. Fisher, A. M., and Scott, D. A., *Biochem. J.*, **29**, 1055 (1935).
10. Eisenbrand, J., and Sienz, M., *Z. physiol. Chem.*, **268**, 1 (1941).

STUDIES ON TISSUE WATER

II. A MACRO MODIFICATION OF THE DISTILLATION METHOD FOR THE DETERMINATION OF TISSUE WATER

By A. T. MILLER, Jr.

(From the Department of Physiology, School of Medicine, University of North Carolina, Chapel Hill)

(Received for publication, May 6, 1943)

In a previous paper[1] the sources of error in the determination of tissue water by the oven-drying technique were pointed out, and a distillation method which avoids these errors was described. The method consisted of reflux distillation of the tissue with toluene, the distilled water falling to the bottom of a tube calibrated for volumetric measurement. The method was devised primarily for determination of the water content of blood and other biological fluids, with samples of 1 ml. or less, and had a reproducibility of 0.2 per cent.

In subsequent work with tissues, in which larger samples were available, it was found that more accurate results could be obtained by a gravimetric modification of the original method. With the modified method, samples of any size may be used, and the receiving tube does not have to be calibrated as in the case of the volumetric tube. Since the details of the macromethod, with the exception of the procedure for measuring the water recovered, are the same as those for the micromethod, only the technique for measuring the recovered water will be described in this paper.

The receiving tube is shown in Fig. 1. It is made of 25 mm. tubing, to the bottom of which is sealed a 2-way stop-cock. The tube is divided into an upper and a lower part by a constriction which at the narrowest point has a bore of 3 to 4 mm. A line etched completely around the tube at this point is used for reading menisci. The total length of the tube is determined by the size of samples to be analyzed.

The procedure for an analysis is as follows: The distillation flask containing the sample and about 100 ml. of toluene (containing 4 per cent *n*-amyl alcohol) is connected to the receiving tube as previously described.[1] The receiving tube is filled from below with mercury to a point just above the beginning of the constriction. Toluene is delivered through the top of the condenser until it reaches the level of the side arm of the receiving tube. The pressure regulator is attached to the top of the condenser and the distillation carried out in the manner described for the micromethod.

After the contents of the receiving tube have cooled to room temperature,

[1] Miller, A. T., Jr., *J. Biol. Chem.*, **143**, 65 (1942).

mercury is delivered from the pipette tip below the stop-cock until the junction of the water and mercury is exactly at the etched meniscus. Mercury is now delivered into a weighing bottle until the junction between water and toluene has fallen to the meniscus. A volume of mercury equal to the volume of water recovered has now been delivered. The mercury is weighed and the weight of water distilled over is calculated by the formula $W = (\text{Hg } V - a + b)\ (D)$, where W = weight of the water, Hg =

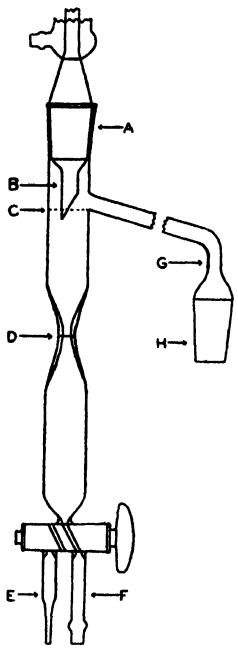


FIG. 1. Receiving tube with condenser tip in place. *A*, "No-lub" joint between condenser and receiving tube; *B*, drip tip sealed to the bottom of the condenser joint; *C*, toluene level during distillation; *D*, etched line for reading menisci; *E*, tip for delivering mercury into the weighing bottle; *F*, tip connecting to the mercury leveling bulb for adjusting the mercury level in the receiving tube; *G*, side arm of receiving tube; *H*, standard taper joint to which the distillation flask is attached.

weight of the mercury, V = volume of 1 gm. of mercury at the weighing temperature (t_w), a = correction for the solubility of amyl alcohol in water (1 per cent of recovery volume), b = volume correction of the apparatus (constant for any given assembly; 0.03 ml. for the apparatus with dimensions described in this paper); D = relative density of water at t_w.

The weight of the water recovered divided by the original weight of the sample gives the percentage of water by weight in the sample.

The reproducibility of results naturally increases with the size of the

sample used. Blank determinations indicate a reproducibility of 0.07 per cent with 5 gm. samples and 0.04 per cent with 10 gm. samples. The absolute accuracy of the method, the distillation time required for different tissues, and general precautions are the same as for the micromethod.

SUMMARY

A macro modification of the distillation method for the determination of tissue water is described. The water is measured by an indirect gravimetric procedure which eliminates the necessity for volumetric calibration of the receiving tube. Samples of any desired size may be used. The reproducibility of the method is 0.07 per cent for 5 gm. samples and 0.04 per cent for 10 gm. samples. Still greater accuracy may be obtained by the use of larger samples when practicable.

FERRITIN

IV. OCCURRENCE AND IMMUNOLOGICAL PROPERTIES OF FERRITIN

By S. GRANICK

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, March 15, 1943)

In 1937 Laufberger (1) reported the presence of ferritin not only in the spleen of the horse but also to a very slight extent in the liver of the horse. Kuhn, Sörensen, and Birkofer, in 1940 (2), then succeeded in finding ferritin in the spleen of the dog, cat, and jackal but were unable to isolate ferritin from the spleen of the guinea pig, rabbit, or whale. These two studies suggested that ferritin might be of wide-spread occurrence. In the present paper a report is made on the occurrence of ferritin in various animal species including the human, and in a number of different organs. Evidence is also presented for the immunological identity of ferritin occurring in different organs of the same animal, but for its specificity as regards the animal species.

Methods

In the isolation of ferritin from different species and organs we have assumed this iron-protein compound to have the properties of solubility and heat stability which characterize horse spleen ferritin. A qualitative notion as to the presence, concentration, and solubility of ferritin in $CdSO_4$ can be obtained by observing the appearance of ferritin crystals in a piece of tissue immersed in 10 per cent $CdSO_4$ solution under a microscope. With a little practice, it is easy to distinguish between tiny crystals and brown hemosiderin granules. In the case of human liver material some protein interferes with the formation of crystals, so that only small brown spheres develop.

In order to compare the yields of ferritin from animal organs of the same species the following procedure was used, which differs in some details from the method previously described (3). The fresh tissue was ground with 1.5 times its weight of water in a Waring blendor, brought to 80°, filtered, and, to every 100 cc. of filtrate, 35 gm. of ammonium sulfate were added, stirred to dissolve, and the material placed in the ice box overnight. The ferritin was crystallized with $CdSO_4$, as described previously (3), except that 48 hours were permitted for crystallization. The material was then recrystallized and after another 48 hours the crystals were centrifuged down at a standardized speed in graduated centrifuge tubes. The yields are expressed as the number of cc. of twice crystallized ferritin isolated from 100 gm. of fresh weight of tissue.

Ferritin Distribution by Isolation Procedure

The distribution of ferritin is presented in Table I. Here we have listed the species and organs from which we have attempted to isolate ferritin. The figures represent the variation in the yield of ferritin observed in a

TABLE I

Ferritin Distribution in Animal Species and Organs As Determined by Isolation Procedure

Species	Organs and tissues examined			
	Spleen	Liver	Red bone marrow	Other organs
Horse	++++ (0.25–0.025)*	+	+++†	Testes +,‡ kidney 0, pancreas 0, brain 0, stomach mucosa 0, striated muscle 0, blood platelets 0, white blood cells 0, red blood cells 0, blood serum 0
Human	++	+++ (0.23 –0.006)	++	
Dog	+	++ (0.039–0.000)	0	Kidney +, pancreas 0, pituitary 0, ovary 0
Guinea pig	+	++ (0.060–0.015)	0	Kidney 0, striated muscle 0, intraperitoneal exudate containing neutrophiles and macrophages, induced by casein, 0
Mouse§	+	+		Testes +,‡ kidney 0, pancreas 0, heart muscle 0
Rat	+	+		Kidney 0
Pig	+	+		Testes +, kidney 0
Rabbit	+	0	0	Testes +‡
Cat	0	0		Kidney +

* The figures in parentheses represent the extremes of ferritin found in these organs, expressed in terms of the number of cc. of packed, twice crystallized ferritin crystals per 100 gm. of the fresh weight of the organ. 1 cc. of packed crystals, dried at 110°, weighs approximately 0.40 gm.

† The ferritin of bone marrow was isolated from the vertebra of a 3 year-old horse and from the manubrium of a 15 year-old horse.

‡ The crystals obtained from the testes of the horse, mouse, pig, and rabbit are always very pale yellow.

§ The distribution of ferritin in mice with inherited lymphocytic leucemia, which were kindly provided by Dr. J. B. Murphy, was identical with that in normal mice.

number of samples, expressed as the number of cc. of twice crystallized ferritin per 100 gm. of fresh weight of tissue, isolated under the conditions mentioned above.

Arranged in the order of decreasing ferritin content the species are horse, human, dog, guinea pig, mouse, rat, pig, rabbit, and cat. We have as yet been unable to find any ferritin in the tissues of cattle, sheep, deer, chick-

ens, or bullfrogs. The organs in which ferritin has been found are the spleen, liver, bone marrow, and kidneys. From the testes of the rabbit, pig, mouse, and horse, colorless crystals of apoferritin were isolated in small yield.

The variation in the amount of ferritin in the same organ of a species may be illustrated with data from normal dog liver and spleen. It will be seen from Table II that there is a great variation in the ferritin content of dog livers, from less than 0.001 to more than 0.1 cc. of crystals per 100 gm. of fresh liver.

The ferritin crystals of all the species from which they have been isolated are optically isotropic (Fig. 1). In the mouse, rat, cat, and pig they appear

TABLE II

Ferritin in Dog Livers and Spleens, Expressed in Cc. of Packed, Twice Crystallized Ferritin

Dog No	Weight of liver	Ferritin in liver		Dog No.	Combined weight of spleens	Ferritin per 100 gm spleen
	gm	*cc per 100 gm*	*cc per liver*		*gm*	*cc*
2	287	0.010	0.030	1, 2	60	Trace
3	250	0.001	0.002	3	20	Absent
4	410	Trace	Trace	4	25	"
5	242	0.012	0.030	5–7	60	0.008
6	250	0.039	0.097	8, 9	30	Trace
7	215	0.116	0.250	10–12	65	0.008
8	210	0.000	0.000			
9	260	0.000	0.000			
10	165	0.022	0.036			
11	185	0.032	0.060			
12	220	0.037	0.082			

as octahedra; in the horse as tetrahedra, octahedra, or twinned octahedra; in the dog as tetrahedra; in the guinea pig as octahedra and as rectangular parallelopipeds; in the human the crystals have rounded edges with a suggestion of an octahedral form, and those obtained from pig testicles are mostly cubes.

Ultracentrifugation studies of apoferritin by Dr. A. Rothen, to be published later, have revealed that the horse, human, and dog apoferritins have approximately the same sedimentation constant and a molecular weight around 500,000. It is possible that apoferritin of other species will also be found to have the same molecular weight.

Immunological Reactions of Ferritin

The question has often arisen whether proteins of one organ are immunologically identical with proteins of another organ from the same

animal. The ordinary proteins prepared, say from the liver or spleen, are not sufficiently characterized to allow of such comparison. On the other hand, such proteins as have been well characterized, as for example pepsin, trypsin, or insulin, appear to be synthesized exclusively by specialized cells of single organs. The occurrence of ferritin in a number of different organs offers a unique opportunity for testing whether crystalline ferritin isolated from one organ is immunologically the same as crystalline ferritin isolated from another organ of the same animal. In addition, if such identity could be established, the precipitin reaction could be used for detecting ferritin in organs in which its content is so low as to make isolation difficult.

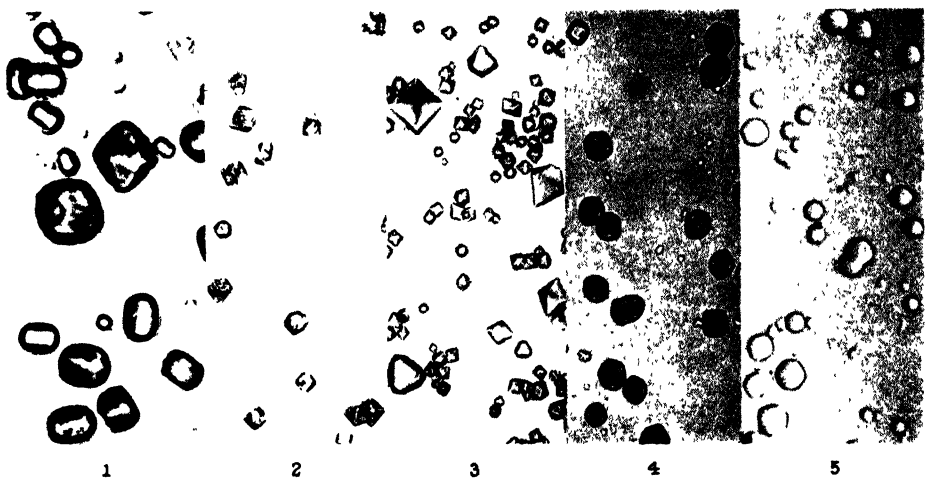


FIG. 1. *1*, guinea pig liver ferritin, × 250; *2*, mouse liver ferritin, × 150; *3*, dog liver ferritin, × 150; *4*, human spleen ferritin, × 150; *5*, human liver apoferritin, × 150.

The antibody against horse spleen apoferritin was prepared by injecting into Rabbit 1 at weekly intervals 7, 14, 21, and 28 mg. of apoferritin, resulting in a serum of low titer. Rabbit 2 was treated similarly except that a final injection of horse spleen ferritin, Preparation XII, was given, resulting in a serum of high titer.

The apoferritin used had been prepared from ferritin Preparation XII by removing the iron according to the procedure previously described (4); it was then subjected to two further crystallizations and finally dialyzed. Ferritin Preparation XII was a preparation that had been crystallized four times and dialyzed.

The absence of antibodies to horse spleen proteins other than apoferritin was examined, since ultracentrifuge studies had shown that horse apo-

ferritin contains a persistent protein impurity amounting to about 1 per cent of the total protein, and since ferritin which possibly was even less pure had been injected into Rabbit 2. For this purpose 2.0 cc. of serum of Rabbit 2 were added to 1.0 cc. of apoferritin (0.7 per cent). An immediate heavy turbidity resulted. After 5 minutes the floccules were centrifuged down, the flocculent precipitate amounting to about 0.10 cc. An additional 0.05 cc. of apoferritin was now added to the supernatant, producing only a trace of cloudiness. After standing overnight in the ice box the tube was centrifuged. The clear supernatant solution resulting was now tested against dilutions of clear horse spleen extract and gave no trace of cloudiness. One may therefore conclude that this rabbit serum is directed primarily against apoferritin.

Tissue extracts for the precipitin test were prepared according to the procedure for the isolation of ferritin, which was followed through the stage at which the ammonium sulfate precipitate containing the ferritin had been centrifuged down. This precipitate was taken up in a small volume of water and dialyzed against isotonic saline until only traces of ammonium ion could be detected. It was centrifuged and the clear supernatant liquid was used for the precipitin tests.

The precipitin tests were conducted in the usual manner with dilutions of the antigen and readings made at appropriate intervals. Only the comparative results are recorded here. Controls were always run with normal rabbit serum or with rabbit immune serum directed against sheep cells. As was to be expected, the antibodies proved to be equally effective in precipitating both ferritin and apoferritin of horse spleen, since the protein constituent is identical. The speed of flocculation with ferritin was somewhat greater than with apoferritin, which may be explained by the greater density of ferritin.

An unexpected result was the relatively strong precipitin reaction given by "non-crystallizable ferritin."[1] The positive precipitin reaction suggested that ferritin was present in this material and the following method was found to permit the isolation of apoferritin as a crystalline Cd salt. 5 cc. of "non-crystallizable ferritin" were placed in a dialysis sac and suspended in a test-tube containing 10 cc. of M/30 phosphate buffer of pH 6.8. A rapid stream of H_2S was bubbled through this outside solution for

[1] The "non-crystallizable ferritin" represents the deep brown soluble material in the mother liquor resulting from the crystallization of ferritin with $CdSO_4$. The brown constituents were concentrated by precipitating with 30 gm. of ammonium sulfate per 100 cc. of liquid, dialyzing, and discarding an insoluble brown residue. This concentrated, soluble brown liquid gives no precipitate with $CdSO_4$ but has a percentage composition not far different from crystallizable ferritin ((3) Table III, Preparation VII).

3 minutes, the pH remaining above 6, and the solution becoming black owing to the formation of FeS. The tube was then stoppered; after 6 hours the sac was removed and dialyzed against distilled water overnight. The contents of the sac were transferred to a centrifuge tube, 50 mg. of ammonium sulfate were added, producing a coagulation of FeS and of protein impurities, and the tube was finally centrifuged. A dark gray supernatant solution resulted which on the addition of 4 cc. of 10 per cent $CdSO_4$ produced pale greenish crystals of apoferritin in a quantity sufficient to account for the strength of the precipitin reaction in "non-crystallizable ferritin." The greenish color of the crystals is probably due to the inclusion of small amounts of iron sulfide in the crystal lattice.

The ferritin crystals isolated from the spleen and vertebral bone marrow of the horse are identical in crystal shape, optical properties, solubility, and heat stability. In the precipitin test they were also found to be immunologically indistinguishable. The relative degree of the precipitin reaction given by extracts of spleen and marrow was also found to be in accord with that expected from the quantities of crystalline ferritin that could be isolated from these tissues. On the basis of the precipitin reaction attempts were made to isolate ferritin or apoferritin crystals from the liver, testes, kidneys, and pancreas. Isolation of crystalline material has been successful in the case of the testicles and the liver. The crystals from these organs were not deep brown but rather pale yellow.

The isolation of ferritin from so many organs suggested that ferritin might have been transported to these organs by the blood stream. However, no trace of ferritin could be demonstrated in the red or white blood cells or blood plasma, although various methods of isolation, fractionation, and concentration were tried on large quantities of these materials. Dr. Rothen called our attention to a protein of horse serum which constitutes about 1 per cent of the globulin and has almost the same sedimentation constant as apoferritin itself. This protein was isolated but did not prove to be apoferritin, since it failed to crystallize out with $CdSO_4$ and gave no precipitin reaction with the antibody.

The organs of the horse listed in the order of their decreasing ferritin content per gm. of moist weight, according to the precipitin test, are spleen, bone marrow, liver, testicles, kidney, adrenal, pancreas, ovary, and lymph nodes. No ferritin was detected in the blood, striated muscle, pituitary, or stomach mucosa.

To determine whether an antibody against horse apoferritin would react with ferritin from other species we have tested crystalline dog and crystalline human apoferritin against the antiserum to horse spleen apoferritin. The dog apoferritin gave a weak precipitin reaction with the horse antibody but the human apoferritin gave no reaction. One may conclude therefore that horse apoferritin is species-specific.

Bleeding in Relation to Iron and Ferritin Content of Horse Spleen

"Normal" horse spleens, obtained from an abattoir, are dark brown in color, owing in part to the high content of ferritin, "non-crystallizable ferritin," and to the presence of a large number of hemosiderin granules. Spleens from horses that have been bled extensively for serum production[2] are bright red in color and have only about one-tenth as much ferritin as the "normal." Our experience with the appearance and ferritin content has been derived from handling some thirty "normal" and seven "bled" spleens. The data presented in Table III illustrate these differences.

TABLE III

Comparison of Spleen of "Normal" and of "Bled" Horses

Source of spleen	Appearance of spleen	Hemosiderin granules	Packed ferritin crystals per pound fresh weight of spleen	Fe in per cent dry weight of spleen
			cc	
Normal horse, 16+ yrs. old, ♀, No. 1128	Dark red-brown	Abundantly present; dense appearance	1.0	1.81
"Bled" horse, 18+ yrs. old, last bleeding 3 mos. before, ♂, No. 972	Bright red	Present in traces	0.10	0.84
"Bled" horse, 23+ yrs. old, last bleeding 17 mos. before, ♂, No. 763	Dark brown	Abundantly present; granules somewhat transparent	1.0	2.10
"Normal" horse, 3 yrs. old	" "	Abundantly present	0.80	

From these data it would appear that extensive bleeding lowers the ferritin, hemosiderin, and total iron content of horse spleen. This suggests that the iron of ferritin and of hemosiderin is utilized in hemoglobin production. The work of Cruz, Hahn, and Bale (5) on radioactive iron has shown that the iron of broken-down erythrocytes is very rapidly utilized in new hemoglobin formation. It is probable that there is some equilibrium between this iron and the storage iron present in ferritin and hemosiderin. A great demand for iron in hemoglobin production, as in the bled horse,

[2] The author wishes to thank Dr. J. Freund of the Research and Antitoxin Laboratory at Otisville, New York, for his very kind cooperation in obtaining various organs and horse serum for us from his laboratory. According to Dr. Freund, horses for serum production are bled every 3 weeks, usually 7.5 liters of blood being taken at one bleeding.

TABLE IV

Ferritin Content of Human Liver and Spleen in Terms of Cc. of Packed Crystals

Autopsy No.	Condition*	Weight of spleen	Ferritin of spleen		Weight of liver	Ferritin of liver	
		gm.	*cc. per 100 gm.*	*cc. per organ*	*gm.*	*cc. per 100 gm.*	*cc. per organ*
10651	Rheumatic heart disease, congestive failure; M., 54	160	0.013	0.021	1100	0.018	0.20
10652	Essential hypertension; M., 41	220	0.033	0.074	1590	0.074	1.28
10653	Intrapartum death; F.	15		Trace	220	0.028	0.063
10655	Leucemia, subacute, lymphoid; M., 43	255	0.00		1840	0.0006	0.011
10656	Coronary occlusion, postoperative inguinal hernia; M., 76	110	0.015	0.017	1600	0.017	0.27
10660	Congenital heart disease; M., 1	24	0.000	No trace	290	0.000	No trace
10664	Born dead (twin); M.				98	0.11	0.11
10673	Mesenteric thrombosis; F., 58	120	0.005	0.006			
10674	Carcinoma of bladder, postoperative shock; M., 62	160	0.028	0.045	1260	0.12	1.53
10677	Carcinoma of bladder, postoperative hematuria, dysuria; M., 44	130	0.100	0.13	2000	0.037	0.74
10682	Hypertensive heart, cardiovascular disease; M., 54	190	0.096	0.18	1530	0.080	1.2
10689	Respiratory failure, postoperative brain tumor; M., 51	170	0.010	0.017	1350	0.006	0.08
10691	Benign tumor of skull; M., 30	210	0.001	0.002	1700	0.036	0.61
10693	Enterocolitis, circulatory failure; M., 3 mos.					0.067	
10694	Cerebral hemorrhage; M., 57	90	0.006	0.006	1360		
10696	Subdural abscess; M., 39	200	0.11	0.22	2130	0.23	4.9
10699	Monocytic leucemia, subacute; F., 31	320	0.10	0.32	1990	0.475	9.45
10700	Essential hypertension, cerebral vascular accident; M., 37	140	0.051	0.071	1800	0.068	1.23
10702	Arteriosclerotic hypertensive heart; M., 83	210	0.058	0.121	2400	0.194	4.67

TABLE IV—*Concluded*

Autopsy No.	Condition*	Weight of spleen	Ferritin of spleen		Weight of liver	Ferritin of liver	
		gm.	*cc. per 100 gm*	*cc. per organ*	*gm.*	*cc. per 100 gm.*	*cc. per organ*
10704	Cirrhosis of liver, alcoholic; fatal gastric hemorrhage; 2 transfusions; M., 48	200	0.022	0.044	1450	0.011	0.161
10709	Acute lymphatic leucemia, infiltration into various organs; 4 transfusions; F., 64	700	0.111	0.78	2100	0.375	7.87
10710	Rheumatic heart disease; F., 41	80	0.0024	0.002	1150	0.104	1.2
10714	Rheumatic heart disease; F., 31	210	0.000		1200	0.0065	0.077

* M., male; F., female; all subjects white. The figure represents age in years except as noted.

would lead to a decrease in the level of ferritin and hemosiderin iron. As for the specific protein apoferritin, its decrease in the bled animal might reflect either a specific drain in connection with the formation of globin and plasma proteins, or might be part of the general drain of protein materials in connection with their formation. A factor possibly suggestive of a more direct rôle played by the specific protein apoferritin in blood metabolism is its relatively high concentration as ferritin in the red bone marrow. For example, the marrow of a normal 3 year-old horse contained about one-fifth of the ferritin content of this horse's spleen per unit of press-juice.

Ferritin in Human

An investigation of human material[3] was undertaken to study the distribution of ferritin in normal cases and to see whether the abnormal cases would suggest any possible function for ferritin. In the human, ferritin was found in the spleen, liver, and bone marrow. Other tissues have not yet been examined for ferritin. Ferritin obtained from humans is difficult to purify, as evidenced by the rounded edges of the crystals even after several recrystallizations, and by ultracentrifuge data on apoferritin. Human apoferritin (Fig. 1) was crystallized with $CdSO_4$ in the presence of acetate buffer of pH 4.1 (ionic strength 0.02). It can be converted back into ferritin by crystallizing with $CdSO_4$ in the presence of the "non-

[3] The autopsy material was provided us through the courtesy of Dr. W. Dock of the Department of Pathology, Cornell University Medical College.

crystallizable ferritin" fraction of the human, or in the presence of the "non-crystallizable ferritin" fraction of the horse.

The apoferritin of humans has a sedimentation constant differing little from that of horse and dog, with a probable molecular weight around 500,000. A comparison of the content of ferritin crystals (Table IV) in spleen and liver indicates that, per unit weight of tissue, liver contains more ferritin than spleen. When one considers the total amount of ferritin in spleen and liver, it may be said that the amount of ferritin in spleen is negligible in comparison with that in the whole liver. When the ferritin content of possibly normal spleen and liver organs (*i.e.* in cases of rheumatic heart disease, hypertension, carcinoma of the bladder, and skull tumors) was averaged, the ferritin isolated was 0.037 cc. of packed ferritin crystals per 100 gm. of spleen (the extremes being 0.000 and 0.110) and for 100 gm. of liver it was 0.067 cc. (extremes, 0.006 and 0.230).

The lowest value of ferritin was obtained in a case of lymphoid leucemia which had followed an acute reaction to arsphenamine. The highest values for ferritin were obtained from a case of monocytic leucemia and one of lymphocytic leucemia in which storage of iron was relatively high, probably due to the inability of bone marrow to function. In a case of cirrhotic liver, the crystals obtained were almost colorless, in contrast to the usual deep brown color in all other cases. On analysis they proved to contain only 5 per cent Fe instead of the usual 23 per cent Fe. It is possible that in liver cirrhosis the iron released from degenerating hemoglobin could not reach the liver directly, where it would customarily be taken up and held by the apoferritin in the cells.

SUMMARY

Ferritin has been crystallized from a number of species and organs and may be considered to be rather widely distributed among mammals. The species from which it has been isolated are, in the order of decreasing ferritin content, horse, human, dog, guinea pig, mouse, rat, pig, rabbit, and cat. The organs from which it has been isolated are spleen, liver, bone marrow, kidney, and testicle. From testicles, crystals are obtained which are pale and contain little or no iron. There appears to be a wide individual variation in ferritin content of an organ of a given species. The crystals of all the species from which they have been obtained belong to the cubic system. Analyses of the ferritin content of the spleens and livers of a number of human cases are also given.

An antibody against horse spleen apoferritin has been produced in the rabbit. With the precipitin reaction it has been found possible to establish that protein crystals of ferritin obtained from one organ are immunologically identical with ferritin crystals obtained from another organ. The

precipitin reaction has also been used as a sensitive test for the detection of apoferritin in various organs of the horse. The organs, listed in the order of their decreasing apoferritin content, are spleen, bone marrow, liver, testicle, kidney, adrenal, pancreas, ovary, and lymph node. No apoferritin could be detected in blood, striated muscle, pituitary, or stomach mucosa. Horse apoferritin antibody reacts slightly with dog apoferritin but not with human apoferritin.

Spleens from normal horses have a relatively high content of ferritin, "non-crystallizable ferritin," and hemosiderin. Those obtained from horses which were bled frequently contain relatively low amounts of these materials, indicating a reutilization for blood production not only of the iron but of the apoferritin as well.

The author desires to express his gratitude to Dr. L. Michaelis for his suggestions and advice in these investigations.

BIBLIOGRAPHY

1. Laufberger, M., *Bull. Soc. chim. biol.*, **19**, 1575 (1937).
2. Kuhn, R., Sorensen, N. A., and Birkofer, L., *Ber. chem. Ges.*, **73 B**, 823 (1940).
3. Granick, S., *J. Biol. Chem.*, **146**, 451 (1942).
4. Granick, S., and Michaelis, L., *J. Biol. Chem.*, **147**, 91 (1943).
5. Cruz, W. O., Hahn, P. F., and Bale, W. F., *Am. J. Physiol.*, **135**, 595 (1942).

THE OXIDATIVE DEAMINATION OF DIAMINES BY HISTAMINASE

By NORMAN R. STEPHENSON

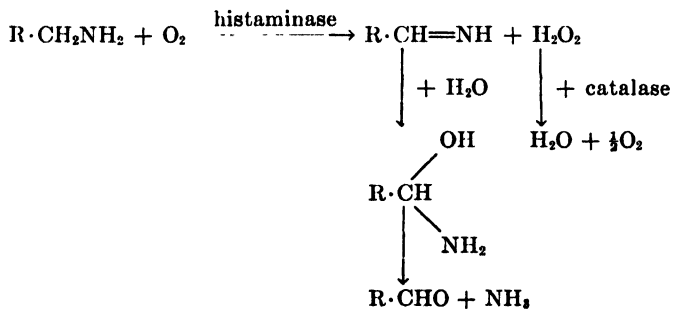
(From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada)

(Received for publication, March 29, 1943)

In 1930, Best and McHenry (1) showed that oxygen is necessary for the destruction of the biological activity of histamine by histaminase, and 2 years later McHenry and Gavin (2) concluded that 1 molecule of ammonia is liberated for every molecule of histamine broken down. Attempts made by Gebauer-Fulnegg and Alt (3) to find a simple stoichiometric relationship between the amounts of oxygen utilized and of histamine inactivated were unsuccessful, but, in 1938, Zeller (4) reported the absorption of 4 to 8 atoms of oxygen per molecule of histamine destroyed. In the following year Zeller, Schär, and Staehlin (5) demonstrated that the biological inactivation of histamine occurred when 1 atom of oxygen was consumed, though their experimental data showed that nearly 2 atoms of oxygen were used in the oxidative deamination. According to Zeller (4), histaminase catalyzes the following reaction.

$$R\cdot CH_2NH_2 + O_2 + H_2O \longrightarrow R\cdot CHO + NH_3 + H_2O_2$$

The extra oxygen absorbed was assumed by Zeller to be due to the further oxidation of the products of the initial reaction. Kiese (6) found that with partially purified extracts of the enzyme approximately 1 atom of oxygen per molecule of histamine was consumed; with less pure extracts, however, 2 atoms were absorbed per molecule of histamine. More recently, Laskowski (7) has reported that the amount of oxygen utilized and the rate of oxygen uptake varied considerably with the purity of the histaminase; his purest preparations catalyzed the consumption of 1 atom of oxygen per molecule of histamine.



The present communication is concerned with a quantitative study of the mechanism of the reaction caused by histaminase when it acts upon histamine, putrescine, and cadaverine. It will be shown that 1 molecule of ammonia is liberated, 1 molecule of aldehyde is formed, and 1 atom of oxygen is utilized per molecule of diamine attacked. Evidence that hydrogen peroxide is probably formed by the system is also included. The whole series of reactions may be represented in the accompanying diagram.

EXPERIMENTAL

Purification of Histaminase—In order to compare the histaminase content of tissues and of extracts of tissues, it is desirable to adopt a standard unit of activity based upon the amount of substrate destroyed under carefully controlled conditions. The unit of histaminase activity arbitrarily adopted is the amount of enzyme necessary to catalyze the consumption of 1 microliter of oxygen in 10 minutes, at pH 7.0 and at 37.5°, in a solution containing 0.1 M ammonium ion and 1 mM histamine, a concentration found to be optimal for small amounts of the enzyme. This unit is equivalent to 1.4 of the units established by Best and McHenry (1). All measurements of oxygen consumption were made with the Barcroft-Warburg apparatus.

The most reliable source of histaminase is hog kidney cortex which contains approximately 150 units of the enzyme per gm. of protein. The active material was extracted from the finely minced cortical tissue of four kidneys with 4 liters of dilute phosphate buffer at pH 7.2. After the removal of inert matter by filtration through cloth, the enzyme was precipitated completely by bringing the extract to 0.5 saturation with ammonium sulfate. A relatively stable powder was prepared by drying this precipitate after most of the ammonium sulfate had been removed by filtration under reduced pressure. The dry powder was extracted with 1 liter of 0.3 saturated ammonium sulfate and the enzyme in this extract was precipitated by adjusting it to 0.55 saturation with ammonium sulfate. The resulting precipitate was treated with 250 ml. of 0.38 saturated ammonium sulfate. The extract obtained after removal of the insoluble matter was brought to 0.55 saturation with ammonium sulfate to precipitate the active material. This final precipitate, when dissolved in phosphate buffer (pH 7.0), usually contained about 1900 to 2000 units of histaminase per gm. of protein and the solution was stable at 5° for at least a week. In the present investigation it was not considered desirable to increase the purity of the enzyme beyond this point owing to the danger of rapid deterioration in solution. Preparations containing 3500 units per gm. of protein were obtained without difficulty, but the activity of the enzyme in solution frequently disappeared in a day or two. On two occasions the activity was increased to approximately 5000 units per gm. of protein by fractionation and precipitation of

histaminase extracts by ammonium sulfate. The purest preparations of the enzyme were obtained in the fraction soluble in 0.4, but insoluble in 0.6 saturated ammonium sulfate. These purified extracts were practically colorless.

Relationship between Substrate Concentration and Oxygen Consumption—To a preparation of histaminase containing approximately 1000 units per gm. of protein, various amounts of histamine were added in order to yield final concentrations in the enzyme-substrate mixture ranging from 0.54 to 10.8 mM. All experiments were conducted at pH 7.0 and 37.5°; the resulting oxygen uptake was measured by means of the Barcroft-Warburg appa-

TABLE I

Relationship between Oxygen Consumption and Concentration of Diamine

Substrate	Concentration of substrate	Total oxygen consumed	Theoretical uptake of oxygen if 1 atom were used per molecule of diamine
	mM	*microliters*	*microliters*
Histamine	0.54	10.5	12
	0.68	15.5	15
	1.35	30.5	30
	2.70	60.0	60.5
	5.40	132.0	121
	10.80	286.0	242
Putrescine	0.65	19.0	16
	1.29	39.0	32
	2.58	69.0	64
Cadaverine	0.56	14.5	14
	1.11	27.5	27.5
	2.22	60.0	55

ratus. The relationship between the oxygen absorbed and the amounts of either putrescine or cadaverine in the enzyme-substrate mixture was also examined. Table I contains a typical set of results from which it is concluded that 1 atom of oxygen is used per molecule of diamine oxidized. However, with higher concentrations of diamine, the duration of the oxidative process is prolonged and slightly more than 1 atom of oxygen per molecule of substrate is consumed. The amount of this additional oxygen uptake increased with larger amounts of substrate and with the length of time required for complete oxygen consumption.

Experiments were also conducted to correlate the amount of oxygen absorbed and the rate of destruction of the biological activity of histamine. Isolated guinea pig ileum suspended in Tyrode's solution at 37.5° was used for the physiological assay of the diamine. It was found that the utiliza-

tion of at least 1 atom of oxygen per molecule of substrate was necessary for the complete disappearance of the contractile effect of histamine.

Formation of Hydrogen Peroxide—So far it has been impossible to demonstrate destruction of a diamine under anaerobic conditions, even in the presence of what should be thermodynamically suitable hydrogen acceptors. Best and McHenry (1) found that histamine was not inactivated by histaminase in an atmosphere of nitrogen. According to experiments performed by Quastel and Wheatley (8) potassium ferricyanide may act as a hydrogen acceptor for certain dehydrogenase systems. It was assumed that hydrogen from the substrate reduces the ferricyanide, releasing hydrogen ions which react with bicarbonate in the solution, causing an evolution of CO_2 which may be measured manometrically. Similar tests, in which ferricyanide was added to the histaminase-diamine system to play the rôle of a hydrogen acceptor, yielded negative results. In addition to ferricyanide, the ability of the oxidation-reduction dyes 2,6-dichlorophenol indophenol, methylene blue, and indigo disulfonate (9) to act as hydrogen acceptors under anaerobic conditions was tested. None of these dyes, including the strongly oxidizing 2,6-dichlorophenol indophenol, changed color after at least 1 hour in the presence of a diamine and 20 units of histaminase under anaerobic conditions. This dye inhibited the enzyme by approximately 20 per cent, but the other dyes had very little effect, if any, on the activity in air. No deamination of the substrate occurred in the presence of a dye and in the absence of oxygen. These experiments suggest that histaminase acts upon diamines only in the presence of molecular oxygen.

If this last statement is true, hydrogen peroxide should be formed during the reaction. Since hydrogen peroxide produced in an oxidase-substrate system may be utilized in coupled oxidations, several methods designed to detect the presence of hydrogen peroxide in an enzyme-substrate mixture have been reported in the literature. For example, hemoglobin was converted to methemoglobin in the presence of the xanthine oxidase system (10) and the amino acid oxidase-substrate mixture (11). Keilin and Hartree (12) found that the addition of either ethyl alcohol to the xanthine oxidase system or *p*-phenylenediamine to the uricase-uric acid mixture utilized an amount of oxygen which was twice that required for the oxidation of the substrate, provided a peroxidase was also present. Zeller (13) reported that methemoglobin was formed when hemoglobin was added to the histaminase-histamine mixture, and also that the presence of ethyl alcohol caused an increase in the amount of oxygen consumed. Since the results of the latter tests did not seem to be entirely conclusive, attempts were made in this laboratory to confirm them.

In these experiments it was possible to detect methemoglobin spectroscopically after histamine had been oxidized by histaminase in the presence

of hemoglobin. In the absence of substrate, the hemoglobin was not oxidized, but the addition of a small amount of 1 per cent hydrogen peroxide caused the immediate formation of methemoglobin. When a few drops of a 1 per cent solution of hydrogen peroxide were mixed with 0.001 M *p*-phenylenediamine, no appreciable color change was detected. However, when 1 ml. of the histaminase extract, which probably contains a factor capable of bringing about coupled oxidations, was added to the mixture, the color of the solution changed from yellow to dark brown. A similar color change was observed when either histamine or putrescine was oxidized by histaminase in the presence of *p*-phenylenediamine. The increase in oxygen up-

TABLE II

Experiments to Illustrate Formation of Hydrogen Peroxide by Histaminase-Diamine Reaction

1 ml. of the enzyme solution + 0.2 ml. of 1 M NH_4Cl (pH 7) + 0.2 ml. of the diamine solution. When the oxidizable material was added, the final volume of the mixture was 2.2 ml.

Oxidizable material	Substrate	Oxygen uptake	
		Without test material	With test material
		microliters	*microliters*
Hemoglobin			2
"	Histamine	41	46
Ethyl alcohol (10 mg.)	"	26	43
" " (10 ")	Putrescine	71	95
Resorcinol (0.01 M)	Histamine	40.5	47
Benzidine (0.001 ")	"	40.5	72.5
p-Phenylenediamine (0.001 M)			0
" (0.001 ")	Histamine	40.5	90

take which occurs when easily oxidizable substances are added to the histaminase-diamine mixture is shown in Table II.

The extra oxygen consumption observed when other oxidizable substances were added to the histaminase-histamine system is most readily explained by assuming that hydrogen peroxide, formed during the oxidative deamination of histamine, causes a secondary oxidation through the action of another enzyme such as peroxidase. Unfortunately, however, no direct evidence for the formation of hydrogen peroxide was obtained.

Formation of Ammonia—McHenry and Gavin (2) and Edlbacher and Zeller (14) showed that 1 molecule of ammonia was released per molecule of histamine inactivated. In 1935, McHenry and Gavin (15) concluded that this ammonia came from the side chain, because a loss in amino nitrogen was detected as the reaction proceeded. It was possible to confirm this

finding by using a copper-iodometric method for the determination of amino nitrogen (16). Typical results are shown in Table III.

If 1 molecule of ammonia were released from the side chain of each molecule of histamine, the ratio of microliters of NH_3 to microliters of O_2 would be 2. Owing to the experimental errors in the methods employed, the ratio is actually between 1 and 2. It shows that in all probability 1 molecule of ammonia is split off from the side chain during the breakdown of the diamine. Zeller (13) reported that 1 molecule of ammonia was liberated per atom of oxygen consumed during the oxidation of histamine.

In order to obtain additional proof of this hypothesis, the relationship between the amount of oxygen absorbed and the quantity of ammonia formed was investigated more thoroughly. After the oxygen consumption was measured, the ammonia content of the reaction mixture in the Barcroft-Warburg vessel was determined. An aliquot of the filtrate, obtained after

TABLE III

Relationship between Loss of Amino Nitrogen and Oxygen Utilized

25 units of histaminase in 5.0 ml. of 6.7 mM histamine.

Time	Oxygen uptake	Amino nitrogen	Ammonia liberated; calculated from loss of amino N	Microliters NH_3 / Microliters O_2
hrs.	*microliters*	*mg.*	*microliters*	
0	0	0.505	0	
1	106	0.415	144	1.36
2.5	270	0.186	510	1.89

the removal of the proteins in the mixture with zinc hydroxide (17), was steam-distilled in the presence of sodium borate in a micro-Kjeldahl apparatus. The amount of ammonia was measured by means of an Evelyn photoelectric colorimeter after nesslerization of the distillate. For histamine the ratio of microliters of NH_3 to microliters of O_2 obtained from twenty-four estimations was 1.58 ± 0.15, while for putrescine, with five determinations, it was 1.91 ± 0.05. The values for putrescine are close to theoretical, while those for histamine show that more than 1 atom but less than 2 atoms of oxygen were utilized per molecule of ammonia liberated. The reason for this discrepancy probably lies in the fact that more secondary oxidation occurs in the presence of histamine than with putrescine. This phenomenon will be discussed in greater detail later.

Detection of Aldehyde As Product of Reaction—Edlbacher and Zeller (14) obtained a crystalline 2,4-dinitrophenylhydrazone from the histaminase-histamine reaction mixture, but were unable to show that it was the hydrazone of imidazole acetaldehyde. In 1940, Zeller, Stern, and Wenk (18) reported that aldehyde was produced during the oxidative deamination

but they did not establish any satisfactory relationship between the amount of aldehyde actually formed and the amount calculated on the basis of Zeller's equation.

In an effort to confirm and extend the finding of Zeller *et al.* (18) with respect to the formation of aldehyde in the reaction, the following experiments were carried out. The enzyme-substrate mixtures were first tested for the presence of reducing substance before and after oxygen consumption had occurred. In every case the reducing power of the mixture, determined according to the Hagedorn-Jensen procedure, increased during the course of the reaction. The bisulfite method of Clift and Cook (19) for the detection of carbonyl compounds was then employed, with certain modifications, to determine the increase in aldehyde content of the reaction mixture after oxygen had been absorbed. With histamine, the average ratio of atoms of oxygen to molecules of aldehyde for ten determinations was 1.11 ± 0.15, while with putrescine it was 0.98 ± 0.05, representing the average of eight estimations. From these results it is apparent that 1 molecule of aldehyde is formed when 1 atom of oxygen is consumed in the oxidative deamination of putrescine. However, the results for histamine show that slightly more than 1 atom is utilized per molecule of aldehyde formed. This extra oxygen uptake is probably due to the oxidation of the product of the reaction.

DISCUSSION

Several authors have observed that more than 1 atom of oxygen is used per molecule of histamine in the reaction mixture (4, 6, 7). However, all agree that with their purified extracts of histaminase, the ratio of atoms of oxygen absorbed to molecules of histamine destroyed is approximately 1. In many of the experiments reported in this paper slightly more than 1 atom of oxygen was consumed per molecule of diamine attacked by the enzyme. This extra oxygen uptake was most apparent in the case of histamine. The other diamines investigated, putrescine and cadaverine, usually behaved in a manner similar to that shown in the equation given above.

When a colorimetric diazo test was applied for the imidazole ring, with nitrosamine red (20), it was found that after 1 atom of oxygen had been consumed per molecule of histamine, the color formed by the product in the reaction mixture was approximately the same as that in the control in which no oxygen had been used. It appears from this experiment that the imidazole ring is not ruptured by the direct action of histaminase as suggested by Best and McHenry (1) but is perhaps oxidized slowly by a coupled oxidation brought about by hydrogen peroxide in the presence of a factor known to exist in tissue extracts (11). This hypothesis is supported by the fact that with histamine the total oxygen consumed is usually 10 to 20 per cent more than the theoretical amount. It also provides a possible explanation for the disappearance of the imidazole ring structure observed in the experi-

ments of Best and McHenry (1), because their tests were conducted over relatively long periods of time.

From the work reported in this paper, it is possible to conclude that histaminase, when in the presence of a diamine, "activates" hydrogen from a terminal amino group and passes it on to molecular oxygen. The imino compound which is assumed to be formed could then take on a molecule of water to form an addition compound. Assuming, further, that this compound is unstable, it would then break down to form 1 molecule of aldehyde and 1 molecule of ammonia. The hydrogen peroxide which is probably formed by the combination of substrate hydrogen with molecular oxygen is destroyed by a factor such as catalase which always accompanies histaminase in the extracts. Accordingly, only 1 atom rather than 1 molecule of oxygen per molecule of histamine is consumed in the reaction.

SUMMARY

1. A method is given for the purification of histaminase from hog kidney cortex.

2. When histaminase acts upon 1 molecule of an aliphatic diamine, 1 molecule of ammonia is liberated, 1 molecule of aldehyde is formed, and 1 atom of oxygen is utilized. Evidence is presented which suggests that hydrogen peroxide is also formed during the reaction.

The author wishes to thank Dr. A. M. Wynne and Dr. B. Mendel for their kind and helpful assistance in this work.

BIBLIOGRAPHY

1. Best, C. H., and McHenry, E. W., *J. Physiol.*, **70**, 349 (1930).
2. McHenry, E. W., and Gavin, G., *Biochem. J.*, **26**, 1365 (1932).
3. Gebauer-Fulnegg, E., and Alt, H. C., *Proc. Soc. Exp. Biol. and Med.*, **29**, 531 (1932).
4. Zeller, E. A., *Proc. XVI Internat. Physiol. Cong., Kongressbericht II*, Zurich, 259 (1938).
5. Zeller, E. A., Schär, B., and Staehlin, S., *Helv. chim. acta*, **22**, 837 (1939).
6. Kiese, M., *Biochem. Z.*, **305**, 22 (1940).
7. Laskowski, M., *J. Biol. Chem.*, **145**, 457 (1942).
8. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **32**, 936 (1938).
9. Oppenheimer, C., and Stern, K. G., Biological oxidation, New York, 52 (1939).
10. Thurlow, S., and Harrison, D. C., *Biochem. J.*, **20**, 217 (1926).
11. Bernheim, F., Bernheim, M., and Gillaspie, A. G., *J. Biol. Chem.*, **114**, 657 (1936).
12. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 141 (1936).
13. Zeller, E. A., *Helv. chim. acta*, **21**, 880 (1938).
14. Edlbacher, S., and Zeller, E. A., *Helv. chim. acta*, **20**, 717 (1937).
15. McHenry, E. W., and Gavin, G., *Biochem. J.*, **29**, 622 (1935).
16. Pope, C. G., and Stevens, M. F., *Biochem. J.*, **33**, 1070 (1939).
17. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
18. Zeller, E. A., Stern, R., and Wenk, M., *Helv. chim. acta*, **23**, 3 (1940).
19. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).
20. Diemair, W., and Fox, H., *Biochem. Z.*, **298**, 38 (1938).

THE EFFECT OF THYROXINE AND DINITROPHENOL ON SPERM METABOLISM*

By HENRY A. LARDY and PAUL H. PHILLIPS

(From the Department of Biochemistry, College of Agriculture, University of Wisconin, Madison)

(Received for publication, March 22, 1943)

In continuation of our studies on the metabolic processes involved in furnishing energy to the mammalian sperm cell a study has been made of the effect of certain metabolic accelerators.

Carter (1) has reported that the respiration of rabbit epididymal spermatozoa may be increased by thyroxine by as much as 200 per cent under certain conditions, and has postulated a rôle of thyroxine in the maturation process of the sperm cell. The work reported here was designed to study the mechanism of the action of thyroxine and dinitrophenol, and an experimental attempt to verify the work of Carter will be reported elsewhere.

Thyroxine and dinitrophenol (DNP) are both known to stimulate the basal metabolic rate but their mechanisms of action are entirely different. The symptoms of myxedema are not alleviated by DNP, even though the metabolic rate is brought to normal. Neither does DNP accelerate developmental processes as does thyroxine (2). DNP has been shown to increase the oxidative metabolism of tissue slices, resting bacteria, and yeast.

The effects of thyroxine and DNP on the metabolism of ejaculated bovine spermatozoa were investigated by the methods used in previous studies of sperm metabolism (3, 4). All experiments were carried out at 37° in the Warburg apparatus. The thyroxine used was the crystalline product of Roche-Organone, Inc.; the DNP was from Eastman. The crystalline orthothyroxine (*dl*-3,5-diiodo-4-(3′,5′-diiodo-2′-hydroxyphenoxy)-phenylalanine) was prepared by Dr. Carl Niemann (5). The thyroxine compounds were dissolved in a minimum amount of dilute NaOH and made up to a dilution of 1:2500 (5.15×10^{-4} M) with redistilled water. In the final sperm suspension in the Warburg flask a concentration of 1:75,000 (1.72×10^{-5} M) was used. No change in pH (glass electrode) of the Ringer-phosphate solution was perceptible on addition of this amount of thyroxine solution.

Results

Dinitrophenol—The effect of various levels of DNP on the endogenous respiration, respiration in the presence of glucose, and aerobic glycolysis of

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the National Committee on Maternal Health, Inc.

spermatozoa is shown in Fig. 1. DNP stimulated glycolysis as well as respiration in the presence of glucose but inhibited endogenous respiration. Maximum stimulation of both aerobic glycolysis and respiration in the presence of glucose was obtained with 1.33×10^{-4} M DNP.

As shown in Table I, DNP stimulated both aerobic and anaerobic glycolysis of bull spermatozoa. In our experience specimens of bull spermatozoa

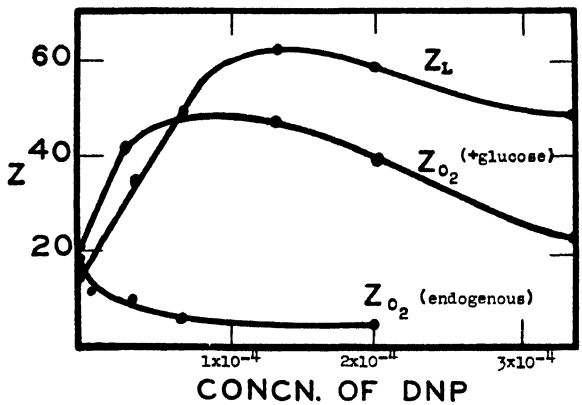


FIG. 1. Effect of various concentrations of DNP on the metabolism of ejaculated bull spermatozoa. Z_{O_2} = c.mm. of O_2 per 100×10^8 cells per hour, Z_L = lactic acid (equivalent to c.mm of CO_2) per 100×10^8 cells per hour (3).

TABLE I

Effect of Dinitrophenol on Glycolysis of Bull Spermatozoa

Bull	Sample	Z_L^{air}	$Z_L^{N_2}$
S	Control	20	25
	+ 0.0001 M DNP	44	58
I	Control	11	21
	+ 0.0001 M DNP	26	31

Semen diluted with an equal volume of Ringer-PO_4 (pH 6.95), centrifuged, and spermatozoa suspended in fresh Ringer-PO_4; glucose added to give a final concentration of 0.02 M; incubated at 37°.

vary a great deal in their exhibition of the "Pasteur effect." The data in Table I include typical results obtained with specimens showing high anaerobic glycolysis (relative to aerobic glycolysis) as well as of specimens showing nearly equal anaerobic and aerobic glycolysis. In all specimens the per cent stimulation obtained by a given concentration of DNP was greater under aerobic than under anaerobic conditions.

The time course of respiration in the presence of glucose and DNP is

shown in Fig. 2. In these experiments glucose and DNP solutions were tipped from side arms into the Warburg flasks after the stop-cocks had been closed. In the presence of glucose only, respiration is linear during the 1st hour. When glucose and DNP were added, only a slight stimulation of respiration occurred during the first 20 minutes but the *rate* of oxygen consumption continued to increase throughout the 1st hour of the experiment. From these results it appeared that either DNP penetrated the spermatozoa slowly and that stimulation increased as the concentration of DNP within the cell increased or that stimulation of respiration could occur only after glycolytic processes had made available substrates for oxidation. To check the latter possibility the effect of DNP on sperm respiration in the presence of pyruvate and lactate was next studied. The results are shown

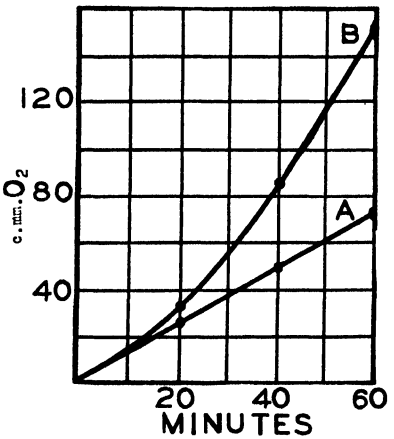


FIG. 2. Effect of DNP on rate of sperm respiration in presence of glucose. Curve A, 0.02 M glucose only; Curve B glucose and 0.00006 M DNP.

TABLE II

Effect of DNP on Sperm Metabolism in Presence of Various Substrates

Sperm in Ringer phosphate +	Z_{O_2}	Z_L^{air}	Motility at 2 hrs.
None	18 7		4+
6.6×10^{-5} M DNP	7 6		Very few motile
0 02 M glucose	17 2	19 8	5+
Glucose and DNP	39 6	31 3	4+
0 02 M lactate	16 1		4+
Lactate and DNP	43 2		2+
0.02 M pyruvate	21 7		5+
Pyruvate and DNP	31 5		2+

in Table II. DNP produced the greatest increase in respiration when glucose or lactate was present and produced an appreciable increase when pyruvate was the substrate. There was no lag period in the stimulation of respiration when pyruvate or lactate was present.

In contrast to its effect on respiration and glycolysis, DNP inhibited motility. In the absence of substrates motility was almost completely inhibited, especially by higher levels of DNP. In the presence of glucose, low concentrations of DNP did not severely inhibit motility but higher concentrations of DNP or prolonged contact with this substance was very detrimental.

The reversibility of the effects of DNP is shown in Table III. The methods used were those developed in studying the reversibility of the effects of metabolic inhibitors on sperm metabolism (4). The inhibition by DNP

TABLE III

Reversibility of Effects of DNP on Metabolism of Bull Spermatozoa

Treatment	Z_{O_2}		Z_L^{air}
	Endogenous	Plus glucose	
(*a*) Control	17.4	17.7	20.4
(*b*) Treated and reversed	13 4	24.4	25 0
(*c*) " " not reversed	5 7	20 6	36 7

(*a*) Held at room temperature during treatment period; (*b*) treated with 2.0×10^{-4} M DNP for 47 minutes, then centrifuged, and the sperm suspended in fresh Ringer-phosphate; no DNP in Warburg flask; (*c*) treated as in (*b*); 2×10^{-4} M DNP in Warburg flask.

of endogenous respiration and motility was largely reversible after 47 minutes treatment with 0.0002 M DNP. After prolonged exposure to DNP the stimulation of respiration in the presence of glucose was slight and it was further increased by removing the spermatozoa and transferring to fresh Ringer-phosphate solution. The stimulation of glycolysis was largely, but not completely, reversible.

Thyroxine—The effect of thyroxine on the metabolism of bull spermatozoa is shown in Table IV. When it was found that thyroxine consistently produced an inhibition of respiration, the effect of orthothyroxine, an isomer of lower activity, was tested. This compound was supplied by Dr. Carl Niemann and has been shown by Boyer, Jensen, and Phillips (6) to have an activity (tested on thyroidectomized rats) of about one-fiftieth that of thyroxine. It was employed to determine whether the effect of thyroxine on sperm metabolism was a general effect of a compound of that type or a specific metabolic effect peculiar to thyroxine only. The ortho compound did not significantly affect the metabolism of the sperm specimens whose

respiration was consistently inhibited and whose glycolysis was consistently stimulated by thyroxine. Neither thyroxine nor orthothyroxine had any appreciable effect on motility at the concentration tested. According to Carter (1) stimulation by thyroxine of the respiration of rabbit epididymal spermatozoa is greatest when the pH of the medium is altered from the optimum. Table V shows the effect of thyroxine on the endogenous respiration of bull spermatozoa in media of various pH values. Thyroxine inhibited respiration in media of optimum pH (6.8 to 7 (3)) as well as in more acid or more alkaline media.

TABLE IV

Effect of Thyroxine on Metabolism of Ejaculated Bull Spermatozoa

Additions	No. of experiments	Average deviation from control			
		Z_{O_2}		Z_L^{air}	$Z_L^{N_2}$
		Endogenous	Plus glucose		
		per cent	*per cent*	*per cent*	*per cent*
Thyroxine 1.7×10^{-5} M	10	−35.9	−19 6	+15.5	+10.7
Orthothyroxine 1.7×10^{-5} M	4	−7 7*	+0.8	+0 4	

* Duplicate control experiments in separate Warburg flasks checked within 8 per cent of each other; hence the difference is not significant.

TABLE V

Relation of pH of Suspension Media to Effect of Thyroxine on Sperm Respiration

pH of medium	Z_{O_2} (endogenous)	
	Control	Plus thyroxine
6.59	12.7	3 8
6.78	17.6	11.1
7.03	16 1	10 4
7.22	16.1	12.4

DISCUSSION

Ditnitrophenol has long been known to stimulate tissue oxidations both *in vivo* and *in vitro*. Clifton and Logan (7) have credited DNP with the property of preventing assimilation of carbohydrate by resting bacterial cells. Burris and Wilson (8) have further shown that DNP will cause assimilated carbohydrate to be oxidized by the bacterial cell. The observation that in spermatozoa DNP stimulates glycolysis, as well as the oxidation of various substrates, would seem to indicate that the stimulation is a general effect on energy-coupled reactions rather than a specific stimulation of fat oxidation, as has been suggested (2). In the ejaculated bull sperm

the endogenous respiration is probably the result of lipid oxidation (9); yet this respiration is inhibited by DNP (Fig. 1, Table II). The fact that DNP decreased the motility of the spermatozoa, while the processes of glycolysis and oxidation are increased, indicates an interference of the energy-coupling mechanism with the result that oxidation and glycolysis run rampant, while the energy is lost as heat rather than as work. Ronzoni and Ehrenfest (10) have found that, in frog muscle, DNP increases the rate of disappearance of phosphocreatine, and that the mechanism for resynthesis of phosphocreatine is less effective under the influence of DNP. Thyroxine depressed respiration and stimulated glycolysis in all specimens of ejaculated bull spermatozoa examined. The effects apparently are specific for this hormone, since the physiologically less active isomer orthothyroxine did not significantly affect sperm respiration or glycolysis.

SUMMARY

Dinitrophenol (DNP) stimulated glycolysis of ejaculated bull spermatozoa and O_2 consumption in the presence of glucose, lactate, and pyruvate, but inhibited endogenous respiration.

Maximum stimulation of both glycolysis and O_2 consumption in the presence of glucose was obtained with 1.3×10^{-4} M DNP.

With glucose in the medium there was a lag period before stimulation of respiration by DNP occurred. No such lag period occurred with lactate or pyruvate as the metabolite.

DNP inhibited sperm motility. The inhibition was greater (*a*) in the absence of added metabolites than in their presence, (*b*) with higher concentrations of DNP, and (*c*) after prolonged contact with the spermatozoa.

Thyroxine in 1:75,000 dilution (1.72×10^{-5} M) inhibited respiration of bull spermatozoa and stimulated glycolysis. Orthothyroxine, an isomer of low physiological activity, did not significantly affect either glycolysis or respiration.

BIBLIOGRAPHY

1. Carter, G. S., *J. Exp. Biol.*, **9**, 378 (1932).
2. Goodman, L., and Gilman, A., Pharmacological basis of therapeutics, New York (1941).
3. Lardy, H. A., and Phillips, P. H., *Am. J. Physiol.*, **138**, 741 (1943).
4. Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **148**, 343 (1943).
5. Niemann, C., and Mead, J. F., *J. Am. Chem. Soc.*, **63**, 2685 (1941).
6. Boyer, P. B., Jensen, C., and Phillips, P. H., *Proc. Soc. Exp. Biol. and Med.*, **49**, 171 (1942).
7. Clifton, C. E., and Logan, W. A., *J. Bact.*, **37**, 523 (1939).
8. Burris, R. H., and Wilson, P. W., *J. Cell. and Comp. Physiol.*, **19**, 361 (1942).
9. Lardy, H. A., and Phillips, P. H., *Am. J. Physiol.*, **133**, 602 (1941); **134**, 542 (1941).
10. Ronzoni, E., and Ehrenfest, E., *J. Biol. Chem.*, **115**, 749 (1936).

ELECTROPHORETIC STUDY OF THE ACTION OF ALKYLBENZENESULFONATE DETERGENTS ON EGG ALBUMIN

By HAROLD P. LUNDGREN, DANIEL W. ELAM,* AND RICHARD A. O'CONNELL

(From the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

(Received for publication, March 11, 1943)

Several phenomena have been described as resulting from the effects of certain synthetic detergents on protein systems. Anson (1) showed that detergents are denaturants. Sreenivasaya and Piric (15) demonstrated that, in the presence of detergents, tobacco mosaic virus dissociated to fragments of smaller size. According to Keilin and Hartree (3) the spectrum of cytochrome *c* is reversibly altered by detergents. Smith (14) observed a dissociation of the chlorophyll-protein complex by sodium dodecyl sulfate. McMeekin (11) used detergent acids as precipitating agents for soluble proteins. The production of fibers from globular protein-detergent mixtures has previously been described (10). Obviously it is of both theoretical and practical interest to determine the nature of the reactions of proteins with detergents.

The present study is concerned solely with an electrophoretic analysis of the reactions of native and denatured egg albumin with detergents of the alkylarylsulfonate type. It will be shown that, in addition to the changes involved in denaturation of the protein, there is formation of complexes between the protein and detergent in solutions alkaline to the isoelectric region.[1] These complexes exhibit well defined electrophoretic boundaries whose mobilities lie between those of the protein and detergent.

The reactions have also been investigated by means of viscosity, diffusion behavior, streaming birefringence, and x-ray analysis, but the results of these studies will be described in a subsequent publication.

EXPERIMENTAL

Egg Albumin—Crystalline egg albumin, a representative corpuscular protein, was prepared from fresh eggs by the method of Kekwick and

* Present address, Shell Development Company, Emeryville, California.

[1] Since this manuscript was written, a paper has appeared by Miller and Andersson (12) on the behavior of native and reduced insulin in Duponol solutions (aliphatic alcohol sulfates of chain length C_8 to C_{18}). Their results indicate that insulin forms complexes with this detergent much in the same manner as egg albumin does with alkylbenzenesulfonates, as found in this investigation.

Cannan (4), crystallized twice, dialyzed until salt-free, and stored in the cold with merthiolate as a preservative. With appropriate concentrations and buffering conditions, the protein gave the correct electrophoretic schlieren patterns, mobilities, and diffusion characteristics (5, 7). The mobility of the descending boundary of a 1 per cent solution in phosphate buffer at pH 6.5 and ionic strength 0.1 was -5.6×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1}. A Tiselius electrophoresis apparatus equipped with the Svensson schlieren optical system was employed.

Heat-denatured albumin was prepared as follows: A 7.5 per cent solution of previously crystallized salt-free albumin titrated to pH 8.0 was heated in a boiling water bath for 10 minutes. The cooled solution was neutralized to pH 6.5, diluted with buffer of pH 6.5 to about 2 per cent protein, filtered through sintered glass to remove particles, and dialyzed in the cold against the buffer. Before electrophoretic analysis, the solutions were adjusted to 1 per cent protein with buffer. Several values of mobility were obtained which appeared to vary with the amount of aggregation which had occurred after denaturation. An average mobility of -6.3×10^{-5} was calculated from the descending boundaries on the heat-denatured preparations in which special care had been taken to avoid aggregation when the solution was adjusted to pH 6.5. This value of mobility for the denatured egg albumin is higher than the value for the native form under similar experimental conditions and it appears from further experiments that the mobility of the denatured form varies, depending on the pH at which the protein was denatured. This is in accordance with the observations of Pauli and Weissbrod (13) and of Bull and Neurath (2) on acid-base changes in egg albumin following denaturation at various pH values. In all probability this accounts for the comparatively large difference in mobility between native and denatured egg albumin noted, whereas Longsworth (7) found the two only slightly different. In the experiments in which detergent was added to the heat-denatured protein, no difficulty was experienced in obtaining solutions having uniform properties.

Alkylbenzenesulfonates—In preliminary studies on the transformation of protein systems by detergents to states favorable for fiber spinning, it was found that the alkylbenzenesulfonates were among the most effective. Among the alkylbenzenesulfonates examined electrophoretically were purified sodium dodecylbenzenesulfonate[2] and decylbenzenesulfonate[2] and two commercial sodium alkylbenzenesulfonate mixtures[3, 4] (Fig. 1).

Each detergent was dissolved in phosphate buffer at pH 6.5 and $\mu = 0.1$.

[2] Monsanto Chemical Company.

[3] Nacconol N. R. S. F., National Aniline Division, Allied Chemical and Dye Corporation.

[4] Atlantic Ultrawet (salt-free), Atlantic Refining Company.

The alkylbenzenesulfonates were highly purified and essentially free of electrolyte impurities. The electrophoretic behaviors were similar for all

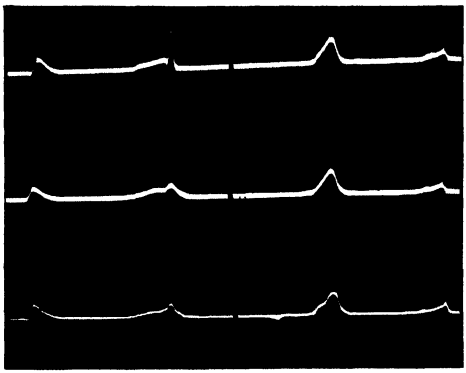


FIG 1. Electrophoretic diagrams of alkylbenzenesulfonates. Top, decylbenzenesulfonate; middle, dodecylbenzenesulfonate; bottom, technical alkylbenzenesulfonate mixture. The ϵ-boundary and the descending boundary are on the right in each diagram. The boundaries on each side of the center in the diagram move toward the center All solutions were at pH 6.5 and 0 1 μ.

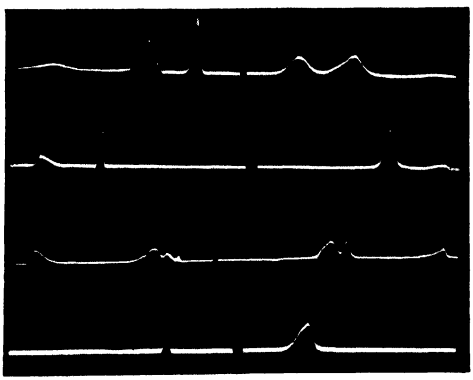


FIG. 2. Electrophoretic diagrams of albumin-alkylbenzenesulfonate mixtures. The weight ratios of native and heat-denatured albumin to alkylbenzenesulfonate in the mixtures were as follows: top, 90:10 native protein-detergent; second, 60:40 native protein-detergent; third, 10:90 native protein-detergent; bottom, 90:10 denatured protein-detergent. The ϵ-boundary and the descending boundaries are on the right in each diagram. The boundaries on each side of the center in the diagram move toward the center. The total protein plus detergent concentration was 1 per cent, pH 6.5, and 0.1 μ.

samples except for one which showed boundary anomalies. The rates of movement of the ascending boundaries were faster than those of the descending boundaries and no attempt was made in this investigation to

calculate the mobilities for the ascending boundaries. The mobilities of the descending boundaries of 1 per cent solutions under these conditions were approximately -20×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1}. The effect of variation in concentration of detergent from 0.25 to 1.5 per cent and in pH from 6.5 to 7.8 showed no detectable difference in the electrophoretic behavior.

Protein-Detergent Systems—Mixtures of the two components were made from 1 per cent solutions at pH 6.5 in phosphate buffer of ionic strength 0.1. The volumes of protein and detergent solutions used in preparing the mixtures were varied from 0 to 100 per cent and from 100 to 0 per cent, respectively. The ratios in which the solutions were mixed will hereafter be referred to as the "mixing" ratios. The mixed solutions were allowed to stand at room temperature for at least 12 hours before analysis, since preliminary runs indicated that the reaction does not approach the final state until after a period of this duration.

Fig. 2 illustrates the typical boundary patterns found in the electrophoretic analysis of these mixtures. Solutions mixed from native protein and detergent in about equal quantities showed only one boundary, indicating a combination of all the protein with all the detergent. The diffusion constant of the species present in these solutions calculated on the basis of diffusion in water at 25° is 5.0×10^{-7} sq. cm. per second, whereas the corresponding value for egg albumin is 7.8×10^{-7} and for alkylbenzenesulfonate is 11.4×10^{-7}. Chemical analysis of the salt-fractionated material from these protein-detergent mixtures shows a definite and reproducible protein to detergent composition and indicates, as do the electrophoretic and diffusion measurements, that a combination occurs between the protein and detergent anions, even though the ascending and descending boundaries are not mirror images. Mixtures made with a large proportion of either native protein or detergent showed two boundaries, suggesting that there are limits in the combining capacity of one component for the other. This is confirmed by chemical analysis of salt-precipitated material from these solutions, to be described later on in this paper. With heat-denatured protein, however, only a single boundary was observed in mixtures containing a large proportion of protein. When a large proportion of the detergent was mixed with either native or heat-denatured protein, a boundary whose mobility corresponded with that of free detergent was observed. Even though the patterns of the ascending boundaries were in general sharper than the descending boundaries (Fig. 2), the observed rates of the ascending boundaries were only slightly different from those of the descending boundaries.

The mobilities of the descending boundaries, expressed in units of sq. cm. sec.$^{-1}$ volt^{-1} $\times 10^{-5}$, are given in Table I as functions of the mixing

ratio. In solutions in which the mixing ratio of native protein to detergent was high, the slower of the two boundaries exhibited a mobility only slightly different from that of native albumin, and was identified by this correlation. Kjeldahl nitrogen analysis of the material recovered from the region in the electrophoresis channel between the two descending boundaries in the case of the mixture having an excess of protein confirmed the mobility measurement by showing the material to be essentially pure protein. The material recovered between the two rising boundaries in solutions containing a high mixing ratio of detergent to protein showed only a trace of nitrogen but was high in sulfur and was therefore practically

TABLE I

Electrophoretic Mobilities of Components in Protein-Detergent System As Function of Mixing Ratio

All experiments were performed in phosphate buffer, μ 0.1, pH 6.5.

Composition		Mobility		
Egg albumin solution	Detergent solution	Egg albumin	Complex	Detergent
vol. per cent	*vol per cent*	$\times 10^5$	$\times 10^5$	$\times 10^5$
0	100			−19.9
10	90		−16.5	−19.4
20	80		−16.1	−18.6
30	70		−15.8	−18.0
40	60		−14.6	−17.7
50	50		−13.8	
60	40		−13.0	
70	30		−12.1	
80	20	−6.2	−10.9	
90	10	−6.0	−10.3	
100	0	−5.6		

pure detergent. In cases in which the detergent was present in excess, its mobility appeared to decrease with increase of the mixing ratio of protein to detergent, suggesting that small amounts of protein might be combined with the detergent. In the absence of accurate information concerning the conductivities at points within the electrophoresis cell, it is difficult to determine the significance of the phenomenon unless it can be ascribed to a further combination of traces of protein with detergent in the faster moving boundary. The main protein-detergent complex always showed a mobility, the value of which lay between those of the single components and varied continuously from one composition to another.

Analysis of Electrophoretic Data—The following method was employed in analyzing the electrophoretic data of the complexes. Empirical con-

stants, K_P and K_D, relating area to concentration for the single components, were obtained from the data on solutions of pure albumin and detergent. It was assumed in calculations of the complex composition that where two peaks occur, one represents the protein-detergent complex and the other excess of a single component, either protein or detergent as the case may be. When the two components were analyzed separately, a significant ϵ-boundary (8) was observed with the detergent; on the other hand the ϵ-boundary of the dialyzed and diluted 1 per cent egg albumin was small enough to be considered negligible for these calculations. Accordingly, in order to measure the concentration of detergent, the area of the ϵ-boundary was added to the areas due to the detergent and was divided in the same proportion as the detergent occurred. The following sample calculations illustrate the method used to determine the percentage of protein in the complex.

Case I. Free Albumin—In this case the percentage of albumin in the complex was

$$\frac{[P_1]}{[P_1] + [D_1]} \times 100 \tag{1}$$

where $[P_1]$ is the amount of albumin in the complex and is equal to $[P] - [P_2]$. In one experiment [P], the total protein concentration used, was 0.91 gm. per 100 cc. and $[P_2]$, the concentration of free albumin, was $K_P A_{P_2} = 0.775 \times 0.826$ or 0.64 gm. per 100 cc. Therefore the amount of combined albumin was 0.91 − 0.64, or 0.27 gm. per 100 cc., but $[D_1]$, the total concentration of detergent used, was 0.09 gm. per 100 cc. Accordingly the albumin in the complex was 75.0 per cent.

Case II. Free Detergent—The percentage of albumin in the complex was

$$\frac{[P]}{[P] + [D_1]} \times 100 \tag{2}$$

where [P] is the total concentration of albumin used and where $[D_1]$ is the concentration of detergent in the complex, or

$$[D_1] = K_D\left[A_{D_1} + A_\epsilon\left(\frac{A_{D_1}}{A_{D_1} + A_{D_2}}\right)\right] \tag{3}$$

A_{D_1} represents the area of the detergent in the complex peak and $A_\epsilon(A_{D_1}/A_{D_1} + A_{D_2})$ is the fraction of the area of the ϵ-boundary corresponding to the detergent in the complex. A_{D_2} represents the area under the peak of free detergent. In one experiment the total concentration of albumin used [P] was 0.1 gm. per 100 cc., all of which was combined with the detergent. 60 minutes after the start of electrophoresis the following areas were measured in arbitrary planimeter units: ϵ-boundary = A_ϵ = 0.281, boundary of the free detergent = A_{D_2} = 0.676, boundary of the

complex = $A_{P_1D_1}$ = 0.233. The total detergent concentration was 0.90 gm. per 100 cc., or $0.90/K_D$, or 1.062 area units where K_D (determined from an analysis of the pure detergent) = 0.847. Therefore the area corresponding to the detergent in the complex A_{D_1} was 1.062 − 0.281 − 0.676 = 0.105. To this must be added $A_\epsilon(A_{D_1}/A_{D_1} + A_{D_2})$, which is 0.039. The percentage of albumin in the complex according to Equation 2 was 45.2.

The relation of the composition of the complex to the mixing ratio as constructed from analysis of the areas is shown in Fig. 3. The data indicate that, if native protein is used to prepare the solution, a maximum of about 75 per cent of protein is present in the complex. With heat-de-

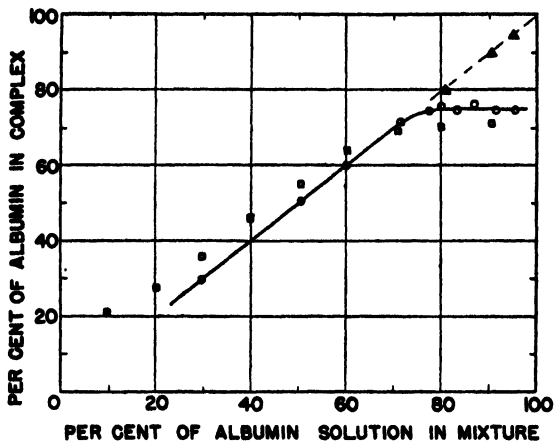


FIG. 3. Relation between composition of protein-detergent complexes and the mixing ratio. Open circles, calculated from electrophoretic data on native albumin-detergent mixtures; squares, calculated from nitrogen analysis of precipitated complex; triangles, calculated from electrophoretic patterns of denatured albumin-detergent mixtures.

natured albumin, there was no similar upper limit. Confirmation of these conclusions was obtained by nitrogen determinations on complexes salted-out of solutions prepared in the same manner. The amount of salt necessary to precipitate the complex is considerably less than that required to precipitate either the protein or the detergent alone. The correlation of the data obtained by two such diverse methods of approach is rather conclusive evidence for the interpretation of the reactions.[5]

[5] The character of the precipitate formed when electrolytes are added to these mixtures is distinct from that of precipitates of either the protein or detergent alone. Aside from being more sensitive to salt, the precipitated complex varies from a floccular to a slimy form as the amount of detergent present increases. The precipitate can be drawn into fibers when it contains over 50 per cent detergent.

Relation of Mobility Data to Composition— In Fig. 4 the mobilities of the protein-detergent complexes are plotted against the mixing ratios of protein and detergent and against the patterns calculated from the electrophoretic composition of the complex. In solutions made up with excess native protein which showed the presence of a free albumin boundary (although

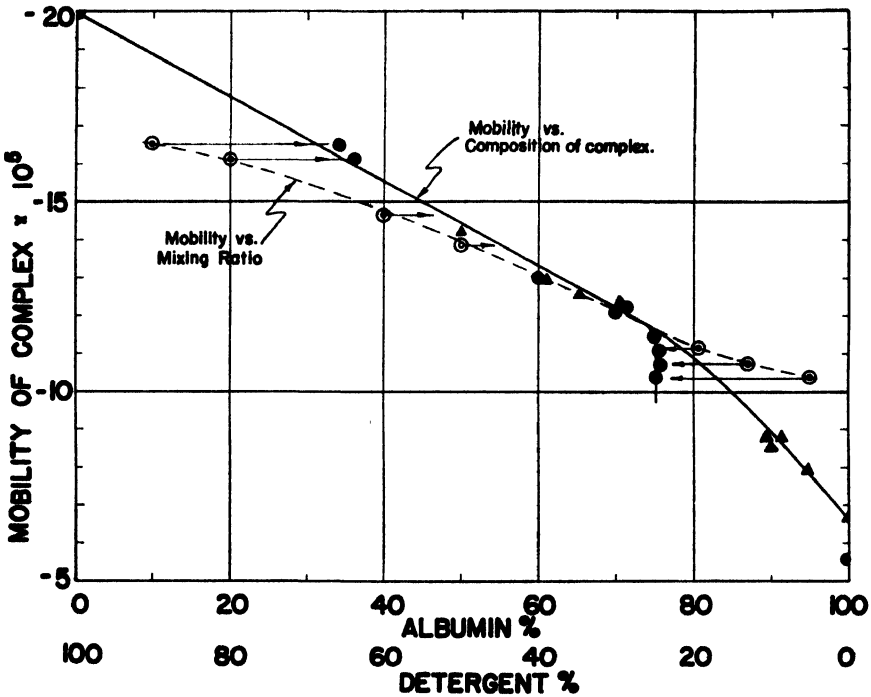


FIG. 4. Relation of mobilities of protein-detergent complex to composition. The mobility of alkylbenzenesulfonate, -20×10^{-5}, is shown at the extreme upper left corner; the mobilities of native albumin, -5.6×10^{-5}, and heat-denatured albumin, -6.7×10^{-5}, are shown below and above, respectively, at the lower right. The same scale is used for the mixing ratio and complex composition. Open circles, mobilities of complexes *versus* mixing ratio; solid circles, mobilities of complexes *versus* composition of complexes; triangles, mobilities of denatured albumin-detergent complexes *versus* composition of complexes. The solid circles on the vertical line at approximately 75 per cent protein represent complexes of native protein.

the composition of the complex remained constant at approximately 75 per cent protein), the mobility of the complex varied with the amount of detergent used. Since the percentage composition of the complex remains constant, the increase in mobility can be interpreted as indicating a liberation of ionizable groups resulting from progressive denaturation of the combined protein.

During electrophoresis of albumin and detergent mixtures, which contain relatively more detergent than a 70:30 albumin-detergent mixture, the area under the peak of the complex grew smaller coincidentally with a gain in the area due to free detergent. With such complexes the mobility value at any one time represents an average of the mobilities of the continuously varying complexes up to the time of the measurement. Accordingly there is an average value of composition whose mobility corresponds to that observed during the entire interval.

This average composition was estimated graphically from the extrapolated curve in which the composition is compared with time. In Fig. 5, such a curve for a 20:80 protein-detergent mixture is shown. It is not

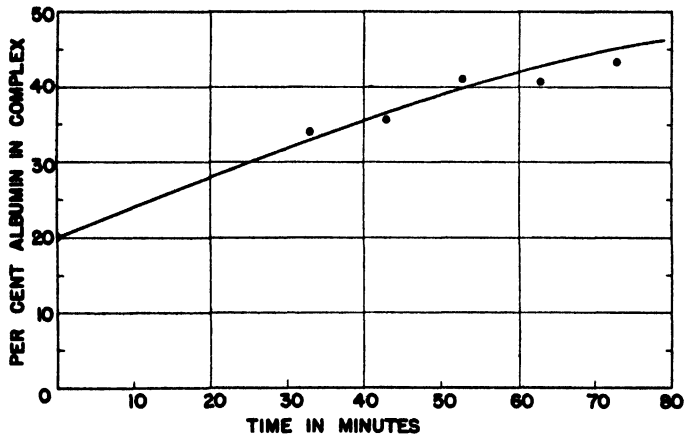


FIG. 5. Composition of protein-detergent complexes of a 20:80 weight ratio of native albumin-detergent mixture at various times during electrophoresis. The curve has been drawn to the original mixing ratio.

certain that the curve may be extrapolated to the original mixing ratio but the error in such an assumption is not significant. The values of the average compositions obtained by dividing the areas under the curve by the time for 10:90 and 20:80 protein-detergent mixtures are shown in Fig. 4. Since the boundaries of free detergent in 30:70 and 40:60 protein-detergent mixtures were very small, the corresponding calculations were not attempted. It is seen that the calculated mobilities of the complexes, within limits, parallel their compositions.

In the experiments just described the total protein-detergent concentration was 1 per cent and the pH 6.5. However, this concentration (90:10 weight fraction of native protein-detergent mixture) could be increased to 4 per cent and the pH to 11.8 without significant difference in distribution of the components, although there were the expected changes in mobility.

DISCUSSION

The reactions which occur when an alkylbenzenesulfonate is added in increasing quantity to a solution of native egg albumin may be described as follows: Up to the value of 0.3 weight fraction of detergent a complex is formed, the composition of which remains constant at approximately 1 part by weight of detergent to about 3 parts by weight of protein, and the excess native protein is detected as a separate boundary.

With heat-denatured protein, however, complexes of continuously varying composition are formed throughout the range of 0 to 0.3 weight fraction of detergent, since only one boundary is observed under these conditions.

To explain why native albumin in this region reacts with a limited amount of detergent, it may be assumed that there is only a definite number of reactive groups structurally available. Upon denaturation more reactive groups are liberated and the protein then reacts in all proportions with the detergent.

With weight fractions of detergent exceeding 0.3 the complexes with native or denatured protein are indistinguishable electrophoretically. The electrophoretic behavior of egg albumin and alkylbenzenesulfonate mixtures is similar to the behavior of mixtures of serum albumin and thymus nucleic acid on electrophoretic analysis as described by Stenhagen and Teorell (16). The interpretation given by Longsworth and MacInnes (9) of their patterns of ovalbumin-yeast nucleic acid mixtures is based on the assumption that a complex is formed that can dissociate both reversibly and rapidly. Under this condition the mobility cannot be determined by means of electrophoresis alone. In the case of the albumin-detergent mixtures, the complexes, after correction for the free detergent, have mobilities proportional to their composition, indicating that the union of the protein and detergent occurs in such a manner that the ionizable groups of both components are additive in their effects on mobility. If the mobilities of the complexes were directly proportional to the composition of the complexes, then the values for the mobilities should fall on a line connecting the mobility of the detergent alone and that of the heat-denatured protein. The values found run somewhat higher and this is taken to favor the view that additional ionizable groups not exposed in the heat-denatured protein are exposed by the action of the detergent.

The nature of the attractive forces between two negatively charged micelles has been discussed (6). Both electrostatic and van der Waals forces have been favored as being responsible for such attractions. Since the ionizable groups of each component appear to contribute to the net charge in the complexes of protein and detergent, the attraction would seem to arise from forces other than those contributed by the ionizable

groups. This conclusion is substantiated by acid-base-combining capacity measurements made on protein-detergent complexes which will be reported in a subsequent publication.

SUMMARY

The results of electrophoretic analysis of an egg albumin-alkylbenzenesulfonate system permit the following conclusions.

1. Both native and heat-denatured egg albumin form complexes of well defined electrophoretic patterns with alkylbenzenesulfonates in solutions alkaline to the protein isoelectric point.

2. In mixtures containing relatively large amounts of native protein, the detergent combines with a maximum of 3 times its weight of protein, leaving the excess native albumin free.

3. When heat-denatured protein is mixed in large amounts with detergent, there is complete combination in all ratios.

4. Complexes formed in solutions containing a large amount of detergent have a correspondingly high detergent content; however, at least some of this detergent is reversibly bound, since dissociation of the detergent from the complex occurs during electrophoresis. This phenomenon occurs in complexes formed by mixing with either native or denatured albumin.

5. The protein-detergent complex exhibits an electrophoretic mobility, within limits, that is parallel to the composition of the complex.

6. The protein in the complex exhibits the electrophoretic behavior of completely denatured protein, when the fraction of detergent present is 0.3 or more.

BIBLIOGRAPHY

1. Anson, M. L., *J. Gen. Physiol.*, **23**, 239 (1939).
2. Bull, H. B., and Neurath, H., *J. Biol. Chem.*, **118**, 163 (1937).
3. Keilin, D., and Hartree, E. F., *Nature*, **145**, 934 (1940).
4. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 227 (1936).
5. Lamm, O., and Polson, A., *Biochem. J.*, **30**, 528 (1936).
6. Langmuir, I., *J. Chem. Physic.*, **6**, 873 (1938).
7. Longsworth, L. G., *Ann. New York Acad. Sc.*, **41**, 267 (1941).
8. Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580 (1940). Longsworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
9. Longsworth, L. G., and MacInnes, D., *J. Gen. Physiol.*, **25**, 514 (1942).
10. Lundgren, H. P., *J. Am. Chem. Soc.*, **63**, 2854 (1941).
11. McMeekin, T. L., *Federation Proc.*, **1**, pt. 2, 125 (1942).
12. Miller, G. L., and Andersson, K. J. I., *J. Biol. Chem.*, **144**, 475 (1942).
13. Pauli, W., and Weissbrod, J., *Kolloid-Beihefte*, **42**, 429 (1935).
14. Smith, E. L., *J. Gen. Physiol.*, **24**, 583 (1941).
15. Sreenivasaya, M., and Pirie, M. W., *Biochem. J.*, **32**, 1707 (1938).
16. Stenhagen, E., and Teorell, T., *Tr. Faraday Soc.*, **35**, 743 (1939).

XANTHURENIC ACID AND ITS RÔLE IN THE TRYPTOPHANE METABOLISM OF PYRIDOXINE-DEFICIENT RATS*

By SAMUEL LEPKOVSKY, ELISABETH ROBOZ, AND A. J. HAAGEN-SMIT

(From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley, and the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

(Received for publication, April 2, 1943)

In a previous publication (1) the isolation of a green pigment from the urine of pyridoxine-deficient rats was described. The green pigment was shown to be the product of a reaction between ferric ammonium sulfate or other ferric salts and a compound whose nature was unknown. This compound has now been isolated in crystalline form. It is a yellow pigment and has been identified as xanthurenic acid.

Isolation of Xanthurenic Acid—The xanthurenic acid was isolated from the urine of pyridoxine-deficient rats with the help of the paper-packed chromatographic column described in a previous publication (1). Cotton pulp has been used successfully and can replace the paper pulp. The yellow pigment was lightly adsorbed from rat urine[1] which had been saturated with sodium chloride. The column was washed with saturated sodium chloride solution, which carried through most of the urinary constituents before the yellow pigment came through. This process was repeated until the yellow pigment was obtained in a fair degree of purity. The sodium chloride-saturated solution containing the yellow pigment was then concentrated to about one-tenth of its volume and the precipitated sodium chloride removed by filtration. At this point, two different procedures were employed.

The solution was further concentrated until, along with sodium chloride, the yellow pigment crystallized out in needles which aggregated to form rosettes. The crystal form of the yellow pigment is illustrated in Fig. 1. The yellow pigment could be separated from the sodium chloride by recrystallization.

The second procedure consisted of eliminating most of the salt from the solution with alcohol and acetone. 2 to 4 volumes of alcohol were added to the salt-saturated solution and to this alcoholic solution was added an equal volume of acetone. The precipitate was discarded and the filtrate was

* This work was supported in part by a grant from the American Cyanamid Company.

[1] Much of the urine from pyridoxine-deficient rats was kindly given to us by the Vitab research laboratory, Emeryville, California.

evaporated in a vacuum until the yellow pigment crystallized out. It was recrystallized from solutions containing small amounts of sodium chloride. Attempts to recrystallize the yellow pigment from distilled water failed.

The yellow pigment differs in many ways from its green iron complex. The green pigment is more readily adsorbed on the paper-packed column than the yellow pigment, and so far it has not been obtained in the form of well defined crystals. The yellow pigment, like the green iron complex, is soluble in strong alcoholic solutions and in alcohol-acetone solutions. These solubility characteristics have been taken advantage of in the isolation of the yellow pigment, as described above.

Identification of Yellow Pigment As Xanthurenic Acid—The elementary analysis of the yellow pigment showed a considerable amount of ash, owing to the presence of sodium. To obtain the free acid, the pigment was dissolved in 50 per cent ethyl alcohol and the acid precipitated with 1 N

FIG. 1. Crystals of the Na salt of xanthurenic acid

H_2SO_4. It was purified further by dissolving in warm 50 per cent alcohol, adding excess 0.1 N NaOH to form the sodium salt, and filtering off the impurities. The acid was reprecipitated with 0.1 N HCl. After two precipitations the melting point, 288°, of the substance could not be raised by further precipitation. The acid, which is a light yellow pigment, changes to yellowish brown in alkaline solution. When an excess of HCl is added, the free acid which is precipitated is redissolved, forming a light yellow-green solution. If the acid solution is left standing or is warmed, it turns nearly colorless. With methyl red it is monobasic; with phenolphthalein, dibasic.

The compound is nearly insoluble in water, slightly soluble in 50 per cent alcohol and in dilute HCl, and easily soluble in alkali hydroxides, carbonates, and hot dilute HCl.

The microanalysis gave the following results.

$C_{10}H_7NO_4$.	Calculated.	C 58.53, H 3.42, N 6.83
	Found.	" 58.27, " 3.70, " 7.12

The equivalent weight was found by titration to be 103.3. Calculated for a dibasic acid of the formula $C_{10}H_7NO_4$ = equivalent weight 102.5.

The analytical data are best explained by assuming that the yellow pigment is the sodium salt of a monohydroxyquinolinecarboxylic acid with 1 molecule of water of crystallization or that of a dihydroxyquinolinecarboxylic acid.

A search of the literature revealed that recently a similar acid had been found by Musajo (2) in urine. This acid is a 4,8-dihydroxyquinoline-2-carboxylic acid and was called xanthurenic acid. The properties of this xanthurenic acid resemble closely those of our yellow pigment.

Since it was not possible to obtain a sample of Musajo's acid and the melting points of kynurenic and xanthurenic acids are close together, and since other properties are also very similar, it was desirable to carry out specific color reactions and prepare the methyl ester for more reliable identification. The methyl ester of kynurenic acid melts at 224°, whereas the methyl ester of xanthurenic acid melts at 262°.

Preparation of Methyl Ester of Xanthurenic Acid—The free acid was suspended in absolute methyl alcohol (3) (25 mg. in 2 cc.). It was heated in a water bath at 60° and dry HCl bubbled through it for 3 hours. When the esterification was completed, the reaction mixture was cooled for 2 hours in ice-salt mixture, centrifuged, and washed twice with cold methyl alcohol. The precipitate was taken up in 25 cc. of boiling water and after being cooled in ice was neutralized with $NaHCO_3$. It was stirred again and allowed to stand overnight under refrigeration. The precipitate was recrystallized from hot 50 per cent ethyl alcohol. After three recrystallizations, 12.4 mg. of methyl ester were obtained with an approximate yield of 50 per cent. It crystallized as light yellow, silky needles which melted at 260–261°. It was dried at 110° and analyzed.

$C_{11}H_9NO_4$.	Calculated.	C 60.25, H 4.14, N 6.40
	Found.	" 60.32, " 4.25, " 6.52[2]

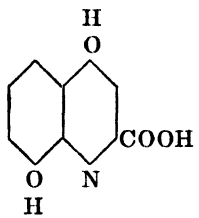
The following color reactions were carried out with the free acid: (1) Millon's reagent, orange-red color; (2) acetic acid-acetic anhydride and benzene, light brown-violet color; (3) freshly synthesized diazobenzosulfonic acid, intense red color.

In order to distinguish between the kynurenic acid and xanthurenic acid, reactions were carried out which gave different colors with these two quinolinecarboxylic acids (4). (1) $FeCl_3$ added in the presence of $NaHCO_3$ gave an intense green color. This is characteristic of xanthurenic acid, whereas kynurenic acid gives no color reaction. (2) Diazobenzosulfonic acid in the presence of $NaHCO_3$ gave the red color of xanthurenic acid. Again kynu-

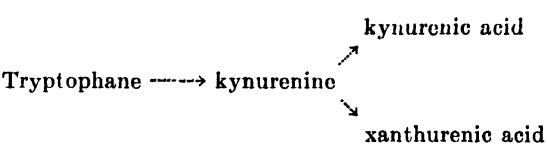
[2] The authors wish to express their thanks to Dr. G. Oppenheimer for carrying out the microanalyses.

renic acid did not give this color reaction. (3) Xanthurenic acid evaporated with concentrated HCl and $KClO_3$ turns brown when moistened with NH_3. Kynurenic acid similarly treated gave a green color. (4) The reaction of Kotake and Shichiri (5); the substance was boiled in alcoholic solution with phenylhydrazine hydrochloride and sodium carbonate and extracted, after being cooled, with ether. On addition of concentrated H_2SO_4, after removal of the ether, no color reaction was observed, whereas kynurenine gives a blue color.

The yellow compound isolated from the urine of pyridoxine-deficient rats must therefore be identical with xanthurenic acid, the formula of which is



Relation of Pyridoxine Deficiency to Protein (Tryptophane) Metabolism – Since xanthurenic acid occurs in the urine of pyridoxine-deficient rats and immediately disappears from the urine on addition of pyridoxine to the diet, it would seem that xanthurenic acid is the result of some sort of metabolic derangement brought about in the rat by inadequate pyridoxine intake. That this metabolic derangement might involve tryptophane metabolism follows from the work of Musajo (6) who found that on high protein diets rats and rabbits excrete both kynurenic acid and xanthurenic acid in the urine. He suggested that these acids found their origin in tryptophane as follows:



If, in pyridoxine-deficient rats, xanthurenic acid originated from dietary tryptophane, the feeding of a tryptophane-deficient diet should result in the disappearance of xanthurenic acid from the urine of pyridoxine-deficient rats and the addition of tryptophane should cause the reappearance of xanthurenic acid in the urine of such rats. This has in fact been found experimentally to be the case.

Pyridoxine-deficient rats were put in metabolism cages[3] and the presence of xanthurenic acid in their urine was established by the production of the

[3] The authors wish to express their thanks to Mrs. Della Parsons for the care of the animals used in this work.

green color with ferric ammonium sulfate. The casein was then removed from their diets and replaced with zein and gelatin, a protein mixture very low in tryptophane. The xanthurenic acid disappeared from the rat urine in from 6 to 12 hours. Each rat was then fed 20 mg. daily of *l*-tryptophane and within 6 to 12 hours xanthurenic acid reappeared in their urine. Addition of 20 γ of pyridoxine[4] daily to the diet of each rat immediately caused the disappearance of xanthurenic acid from the rat urine.

This experiment was repeated with acid-hydrolyzed casein replacing the casein in the pyridoxine-deficient diet. The absence of tryptophane in the acid-hydrolyzed casein was established by qualitative tests. The results obtained with this tryptophane-deficient diet were identical with those obtained with the use of zein and gelatin as the tryptophane-deficient proteins. This leaves little doubt that the function of pyridoxine is related to tryptophane metabolism.

These results are in harmony with those of Voris and Moore (7) who showed that pyridoxine is related to protein metabolism. They used paired rats, one rat of each pair receiving pyridoxine. The pyridoxine-fed rat gained more weight than its paired pyridoxine-deficient mate, and the gains in weight were exclusively protein, whereas in similar studies with thiamine and riboflavin the gains were represented by fat.

Foy and Cerecedo (8) studied pyridoxine deficiency in rats on diets containing 15, 30, and 45 per cent casein. At the low level of protein intake, little dermatitis developed in 70 days, while at the intermediate level the rats developed dermatitis after 30 days. At the highest level of protein intake (45 per cent casein) severe dermatitis developed in 26 days followed shortly after by death. Apparently high protein (tryptophane?) intake seems to increase the severity of the nutritional disorder in rats due to inadequate intake of pyridoxine. Perhaps the excess protein destroys pyridoxine in the rat in some unknown way. Such a phenomenon would explain the early appearance of xanthurenic acid in the urine of rats and rabbits (6) fed high protein diets.

Species Differences– While pyridoxine-deficient rats excrete an abundance of xanthurenic acid in their urines, pyridoxine-deficient dogs excrete very little (9), even when they have become very anemic as a result of the deficiency. So far, no xanthurenic acid has been demonstrated in the droppings of chicks.[5] Wintrobe *et al.* (10) have reported the occurrence of a compound in the urine of pyridoxine-deficient pigs which gives a green color with the addition of ferric ammonium sulfate, indicating the presence of xanthurenic acid.

[4] The pyridoxine was generously supplied by Merck and Company, Inc., Rahway, New Jersey.

[5] Kratzer, F. H., unpublished results.

DISCUSSION

In the past, three related compounds, namely kynurenic acid, kynurenine, and xanthurenic acid, have been isolated from urine as products of protein metabolism. Kynurenic acid was first isolated by Liebig (11) in 1853, and Ellinger and Matsuoka (12) found it after feeding tryptophane. Kotake and Iwao (13) isolated kynurenine as an intermediary product in tryptophane metabolism and reported that kynurenine is excreted as a potassium salt, whereas kynurenic acid is found as a sodium salt. Musajo (2) found a compound similar to kynurenic acid in connection with feeding high protein diets and named it xanthurenic acid.

The excretion of kynurenic acid, kynurenine, and xanthurenic acid seems to be connected with vitamin deficiencies. Rabbits on a diet of polished rice, which is deficient in the vitamin B complex, excrete not only kynurenic acid but also kynurenine (13). In this paper it is shown that the urine of rats on a diet deficient in pyridoxine contains xanthurenic acid. Whether other members of the vitamin B complex are also related to tryptophane metabolism remains to be shown. Thus far all that is known is that pantothenic acid-deficient rats do not excrete any xanthurenic acid in their urine.

The species differences, especially as found with the dog and rat, are of interest. Pyridoxine-deficient rats excrete an abundance of xanthurenic acid in their urine, but they do not become so severely anemic as pyridoxine-deficient dogs (9); yet such dogs excrete but very little xanthurenic acid (9). It must follow, therefore, that the metabolism of tryptophane does not follow the same pathway in both the rat and the dog.

SUMMARY

1. A yellow compound was isolated from the urine of pyridoxine-deficient rats.

2. This yellow compound is shown to be identical with Musajo's xanthurenic acid, a 4,8-dihydroxyquinoline-2-carboxylic acid.

3. Xanthurenic acid was shown to originate in dietary tryptophane.

4. The relation of pyridoxine to tryptophane and protein metabolism has been discussed.

BIBLIOGRAPHY

1. Lepkovsky, S., and Nielsen, E., *J. Biol. Chem.*, **144**, 135 (1942).
2. Musajo, L., *Chem. Abst.*, **29**, 7347 (1935); *Atti accad. Lincei*, **21**, 368 (1935).
3. Gordon, W. G., Kaufman, R. E., and Jackson, R. W., *J. Biol. Chem.*, **113**, 125 (1936).
4. Musajo, L., *Chem. Abst.*, **31**, 7060 (1937); *Gazz. chim. ital.*, **67**, 165 (1937).
5. Kotake, Y., and Shichiri, G., *Z. physiol. Chem.*, **195**, 158 (1931).
6. Musajo, L., *Chem. Abst.*, **29**, 6292 (1935); *Boll. Soc. ital. biol. sper.*, **10**, 290 (1935).
7. Voris, L., and Moore, H. P., *J. Nutrition*, **25**, 7 (1943).

8. Foy, J. R., and Cerecedo, L. R., Abstracts, Division of Biological Chemistry, American Chemical Society, Atlantic City, Sept. 8 (1941).
9. Fouts, P. J., and Lepkovsky, S., *Proc. Soc. Exp. Biol. and Med.*, **50**, 221 (1942).
10. Wintrobe, M. M., Follis, R. H., Jr., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Bull. Johns Hopkins Hosp.*, **72**, 1 (1943).
11. Robson, W., *J. Biol. Chem.*, **62**, 495 (1924–25).
12. Ellinger, A., and Matsuoka, Z., *Z. physiol. Chem.*, **109**, 259 (1920).
13. Kotake, Y., and Iwao, J., *Z. physiol. Chem.*, **195**, 139 (1931).

SERUM PHOSPHORUS CHANGES DURING THE ABSORPTION AND METABOLISM OF GLUCOSE, GALACTOSE, AND XYLOSE*

By ALFRED H. FREE and JACK R. LEONARDS

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

(Received for publication, April 14, 1943)

Since Fiske (1) first pointed out that the ingestion of sucrose by human subjects results in a decrease in the rate of excretion of urinary phosphate, many studies have been made of the changes in blood phosphorus after the ingestion or injection of glucose (2 9). However, very little attention has been paid to the changes in serum phosphorus after ingestion of other sugars such as galactose or xylose. Koehler, Rapp, and Hill (10) found that ingested lactose caused a decrease in the inorganic phosphate of whole blood but McCullagh and McCullagh (11) attribute this fall to the glucose, since in one patient they found galactose to have no effect on serum inorganic phosphorus. Barrenscheen and coworkers (12) found a small irregular decrease in serum inorganic phosphorus after ingestion of 40 gm. of galactose by three patients but no effect on the urinary excretion of phosphate was noted in their experiments.

The following experiments were carried out to obtain further data on the relation of serum inorganic phosphate to the absorption and metabolism of galactose and xylose in normal human subjects and in normal dogs.

Methods

In the ingestion experiments with both humans and dogs 0.6 gm. of the sugar per kilo of body weight was administered as a 10 per cent solution. The solution was flavored with lemon juice for the oral administration to humans and in the studies with dogs the solution was administered by stomach tube. In the other experiments the same amount of the sugar in a sterile 10 per cent solution was injected intravenously at a uniform rate over a period of 1 hour. This method of gradually administering the sugar more nearly simulates the rate at which the sugar enters the blood stream from the intestine than if it were rapidly injected. Urine samples were collected for 1 hour before the test dose and hourly thereafter except in the studies in which galactose was given orally, when the periods were of 90 minutes duration. A sample of blood was obtained during fasting in all

* This investigation was supported by a grant from the Williams-Waterman Fund of the Research Corporation. Presented before the Division of Biological Chemistry of the American Chemical Society at Buffalo, September, 1942.

cases and subsequent samples were taken at half hourly periods in the human studies and more frequently in the canine experiments. The blood samples obtained for analysis during the injection period were from a region of the body other than that in which the injection of sugar was being made. Glucose was determined in oxalated whole blood by the method of Hagedorn and Jensen (13) following precipitation of the proteins with tungstic acid. Galactose and xylose were determined by the same method following

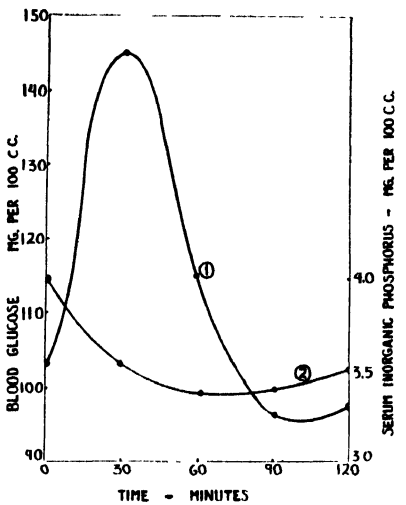


FIG. 1

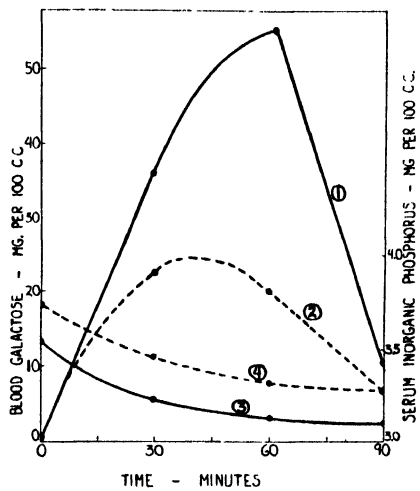


FIG. 2

FIG. 1. Effect of the oral administration of glucose (0.6 gm. per kilo of body weight) to humans on the blood glucose (Curve 1) and serum inorganic phosphorus (Curve 2). The results are averages obtained on eight normal subjects.

FIG. 2. Effect of the oral and intravenous administration of galactose (0.6 gm. per kilo of body weight) to humans on the serum inorganic phosphorus and blood galactose. Curve 1 represents blood galactose after the sugar is given intravenously; Curve 2, after oral ingestion. Curve 3 represents serum inorganic phosphorus after the sugar is given intravenously; Curve 4, after oral ingestion. The results are averages obtained on eight normal subjects.

the removal of glucose from the blood by fermentation with bakers' yeast (14). Serum inorganic phosphate was determined by the method of Fiske and Subbarow (15) adapted for use with an Evelyn photoelectric colorimeter. In all cases the results are recorded as mg. of phosphorus per 100 cc. of blood serum. The normal human subjects used in this study were all male medical students.

Results

Fig. 1 shows the average changes in blood glucose and serum inorganic phosphorus in eight human subjects after the ingestion of glucose. The

changes in blood glucose are typical of those observed in a normal test of glucose tolerance. The serum inorganic phosphorus decreased from 3.9 mg. per 100 cc. to a minimum of 3.3 mg. per 100 cc. at 60 minutes. The urinary excretion of phosphorus decreased from an average control value of 40 mg. per hour before the test to 30 mg. during the 1st hour after glucose ingestion and 14 mg. during the 2nd hour.

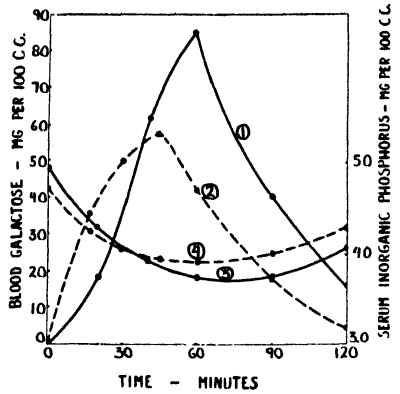


FIG. 3

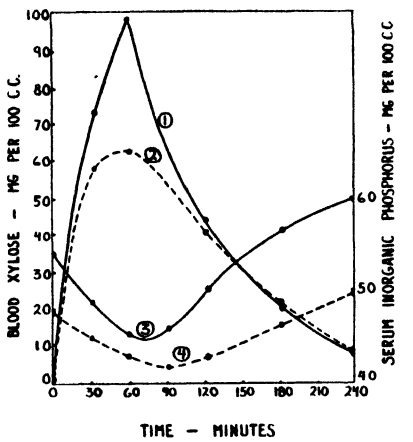


FIG. 4

FIG. 3. Effect of the oral and intravenous administration of galactose (0.6 gm. per kilo of body weight) to dogs on the serum inorganic phosphorus and blood galactose. Curve 1 represents blood galactose after the sugar is given intravenously; Curve 2, after ingestion. Curve 3 represents serum inorganic phosphorus after the sugar is given intravenously; Curve 4, after ingestion. The results are averages for twelve dogs receiving the sugar orally and six intravenously.

FIG. 4. Effect of the oral and intravenous administration of xylose (0.6 gm. per kilo of body weight) to dogs on the serum inorganic phosphorus and blood xylose. Curve 1 represents blood xylose after the sugar is given intravenously; Curve 2, after ingestion. Curve 3 represents serum inorganic phosphorus after the sugar is given intravenously; Curve 4, after ingestion. The results are averages for five dogs receiving the sugar orally and two intravenously.

Fig. 2 shows the average changes in serum inorganic phosphorus and blood galactose when the sugar was given both orally and intravenously to the same eight subjects who received the glucose in the preceding experiments. The intravenous administration of galactose gave a somewhat higher blood galactose level than did the ingestion of the sugar. Similar decreases in serum inorganic phosphorus were obtained following administration of the sugar by either route. The urine phosphorus was 51 mg. for a 90 minute control period and decreased to 38 mg. for a 90 minute period after the ingestion of galactose. When the galactose was administered intravenously, there was a similar decrease in the excretion of urine

phosphate from 24 mg. per hour in the control period to 14 mg. in the 1st hour and 12 mg. in the 2nd hour after the injection was started. Although the values given in Fig. 2 represent averages for the whole group, it is significant that in every individual case there was a decrease in serum inorganic phosphorus and a decrease in urine phosphorus.

Fig. 3 shows the changes in serum inorganic phosphorus and blood galactose following the oral and intravenous administration of galactose to normal female dogs. The decrease in serum inorganic phosphorus after the ingestion of galactose is somewhat less than after the intravenous administration of the sugar.

The effects of the oral and the intravenous administration of xylose to normal dogs are shown in Fig. 4. When the xylose was administered orally, the level of the blood xylose reached a maximum in 60 minutes, and after this time the blood concentration decreased at a much slower rate than with galactose. When the sugar was given intravenously, the rate of xylose removal from the blood was also slower than that of galactose. The serum inorganic phosphorus decreased after the sugar was given by both routes but the decrease was somewhat greater after the intravenous administration. The maximum decrease occurred at 60 minutes when the sugar was given intravenously and at 90 minutes after the oral administration. The level of serum phosphorus then increased and attained a higher concentration than was present in the original fasting sample.

DISCUSSION

It is significant that in all of the forty-one experiments reported a decrease in the serum inorganic phosphate resulted after the administration of the sugar. As a control experiment similar volumes of physiological saline were administered both orally and intravenously to dogs and no changes of serum inorganic phosphorus resulted.

During the past two decades in which it has been known that serum inorganic phosphorus decreases after sugar ingestion, a vast amount of knowledge has accumulated concerning the relationships of phosphorus-containing compounds to intermediary carbohydrate metabolism (16–18). The principal reasons for the changes in serum phosphorus following the administration of sugars are at present obscure. The various possibilities that are suggested include the utilization of serum phosphorus in (1) phosphorylation of the sugar during absorption, (2) the formation of liver glycogen, (3) the formation of muscle glycogen, (4) muscle glycolysis, (5) phosphorylation of sugar during tubular reabsorption in the kidney, and (6) excretion of phosphorus in the urine. In the present studies and also as observed by other investigators the urinary excretion of phosphorus decreased after the administration of glucose and galactose. This indicates

that the level of serum phosphorus governs the urinary excretion rather than being dependent on it. It has been suggested that tubular reabsorption of sugar in the kidney involves a process of phosphorylation (16, 19). In consideration of the effect of tubular reabsorption, we have noted decreases in serum phosphorus after oral and intravenous administration of galactose or xylose to a nephrectomized dog, which were similar to the decreases observed in normal dogs. In other experiments in which the urinary excretion of galactose was markedly increased owing to a liver impairment there were also similar changes in serum inorganic phosphorus. It appears that the process of tubular reabsorption has very little if any effect in decreasing serum inorganic phosphorus after carbohydrate administration.

The experiments in which changes in serum phosphorus were compared after oral and intravenous administration were designed to determine whether the passage of the sugar across the intestinal mucosa was in any way responsible for these changes. The results of the experiments shown in Figs. 2 to 4 indicate that the decrease of serum phosphorus tends to be greater after intravenous than after oral administration. There are certain dissimilarities in rates of administration by oral and intravenous routes that cannot be avoided and that might account for the small differences in the serum phosphorus curves. However, it appears that the process of the absorption of galactose or xylose has no effect on the decrease in serum phosphorus.

In the experiments on humans the decrease in serum phosphorus after galactose by either the oral or intravenous route was of the same character as the decrease in serum phosphorus after a similar quantity of glucose given orally. At the present time the relative rôles of the liver and muscles in removal of galactose from the blood are still uncertain. Therefore on the basis of our experiments it is impossible to determine by which mechanism the serum phosphorus is decreased after administration of the sugar.

The observation that the administration of xylose results in similar decreases in serum phosphorus strongly suggests that it is utilized in a manner which is similar to that of glucose and galactose. However, since the decrease in serum phosphorus is somewhat less after xylose than after galactose administration and also since the rate of removal of xylose from the blood is slower, it appears that the rate of utilization of xylose is less than that of glucose or galactose. Similar observations have been noted in a nephrectomized dog in which the removal of xylose from the blood must have been due to a utilization of the sugar.

SUMMARY

1. The changes in blood glucose and serum inorganic phosphorus in eight normal human subjects were determined at intervals following the ingestion

of 0.6 gm. of glucose per kilo of body weight. Similar studies were carried out after oral and intravenous administration of the same quantities of galactose. A decrease in serum inorganic phosphorus and urinary excretion of phosphorus occurred in all of these studies.

2. Serum inorganic phosphorus decreased following the oral and intravenous administration of 0.6 gm. per kilo of body weight of galactose or xylose to normal dogs.

3. Comparison of the changes of serum phosphorus following the oral and intravenous administration of galactose and xylose indicates that the process of intestinal absorption of the sugar does not influence the decrease in serum phosphorus.

4. Indirect evidence is presented to show that xylose is metabolized by the dog, although the rate of metabolism is slower than that of glucose or galactose.

BIBLIOGRAPHY

1. Fiske, C. H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **41**, p. lix (1920).
2. Katayama, I., *J. Lab. and Clin. Med.*, **11**, 1024 (1926).
3. Harrop, G. A., Jr., and Benedict, E. M., *J. Biol. Chem.*, **59**, 683 (1924).
4. Blatherwick, N. R., Bell, M., and Hill, E., *J. Biol. Chem.*, **61**, 241 (1924).
5. Bolliger, A., and Hartman, F. W., *J. Biol. Chem.*, **64**, 91 (1925).
6. Hartman, F. W., and Foster, D. P., *Am. J. Clin. Path.*, **2**, 289 (1932).
7. Sokhey, S. S., and Allan, F. N., *Biochem. J.*, **18**, 1170 (1924).
8. Reiser, R., *J. Biol. Chem.*, **135**, 303 (1940).
9. Pollack, H. R., Millet, R. F., Essex, H. E., Mann, F. C., and Bollman, J. L., *Am. J. Physiol.*, **110**, 117 (1934).
10. Koehler, A. E., Rapp, I., and Hill, E., *J. Nutrition*, **9**, 715 (1935).
11. McCullagh, E. P., and McCullagh, D. R., *J. Lab. and Clin. Med.*, **17**, 754 (1932).
12. Barrenscheen, H. K., Doleschall, F., and Popper, L., *Biochem. Z.*, **177**, 67 (1926).
13. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).
14. Beams, A. J., Free, A. H., and Glenn, P. M., *Am. J. Digest. Dis.*, **8**, 415 (1941).
15. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
16. Kalckar, H. M., *Chem. Rev.*, **28**, 71 (1941).
17. Cori, C. F., Biological symposia, Lancaster, **5**, 131 (1941).
18. Lipmann, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1**, 99 (1941).
19. Kalckar, H., *Skand. Arch. Physiol.*, **77**, 46 (1937).

LIVER GLYCOGEN AND LIPIDS IN FASTED AND GLUCOSE-FED RATS

By C. R. TREADWELL, HERBERT C. TIDWELL, AND BARNEY G. GRAFA, JR.

(From the Department of Biochemistry, College of Medicine, Baylor University, Dallas)

(Received for publication, April 19, 1943)

Deuel and Davis (1) and Treadwell *et al.* (2) have shown that rats receiving a diet high in fat and low in protein and lipotropic factors have a decreased tolerance for glucose. In this laboratory (2), the livers of these animals (unfasted) contained significantly greater amounts of glycogen and fat than the livers of rats which received a diet high in fat and protein. Thus, while the decreased tolerance could not be related to a loss of ability by the fatty liver to store glycogen, it was suggested that the excessive lipid material present may have affected the rate at which the glycogen was stored and removed. MacLean, Ridout, and Best (3) have found that there was less glycogen stored in very fatty livers than in the livers of choline-fed animals when a certain quantity of sugar was absorbed.

In the present communication we are reporting the results of experiments designed to throw additional light on the relationship of liver glycogen and fat to glucose tolerance in animals with fatty livers. In Series A, the changes in the levels of glycogen and total lipids in the livers of male rats have been followed during fasting and after the administration of a standard dose of glucose. The observed changes in the level of glycogen have been taken as a measure of the rate of deposition and removal. In Series B, the observations were repeated on unfasted females and females fasted 24 hours. The effect of three hourly doses of glucose on the liver glycogen was also determined.

Procedure

The experimental animals of Series A were male rats of the Wistar strain obtained from a commercial source. In Series B, female rats of the strain maintained at the Scripps Metabolic Clinic[1] were used. For 1 week after arrival in the laboratory the animals received Purina dog chow and distilled water *ad libitum*. The initial weight of the males averaged 123 gm. with a range of 110 to 140 gm. The females averaged 151 gm., with a range of 130 to 170 gm. During the experimental periods all animals of each series received daily the same amount of the diets as was ingested by the rat having the lowest food intake the previous day. This procedure was adopted to secure a more constant fat and glycogen content of the liver

[1] We are indebted to Eaton M. MacKay for the gift of these animals.

at the start of the fasting periods. Diet 1 contained 5 per cent casein and 48 per cent cerelose;[2] Diet 2, 30 per cent casein and 23 per cent cerelose; Diet 5, 5 per cent casein, 1 per cent choline, and 47 per cent cerelose. All three diets contained 2 per cent agar, 5 per cent salt mixture (4), and 40 per cent lard. Each animal received 1 dried yeast pill (389 mg.) and 2 drops of cod liver oil per day. The room temperature was maintained between the limits of 23° and 27°.

Fasting and Glucose Administration—To obtain comparable feeding habits and to determine more accurately the start of the fasting periods, the food cups were removed from the cages between 2.00 and 2.30 p.m. and returned between 4.00 and 4.30 p.m. On the day the fasting periods commenced the cups were not returned to the cages and 4.00 p.m. was considered the start of the fast. In Series A, a single dose of glucose (3.5 gm. per kilo) was given at the end of the 24 hour fast. In Series B, animals fasted 24 hours received three of the standard doses 1 hour apart and were sacrificed at the end of the 3rd hour. The glucose was administered as described by Cori (5) and the completeness of absorption checked according to the directions of Deuel *et al.* (6). In view of the recent findings of MacKay and Clark (7) that the volume in which glucose is administered may influence the rate of absorption, the sugar was given in a total volume of 2 cc.

Liver Glycogen and Lipids—The livers were removed from the animals under sodium amytal anesthesia and frozen with carbon dioxide snow. The frozen tissue was reduced to a powder with a crushing machine and transferred to a tared flask containing hot 30 per cent potassium hydroxide. The methods for the determination of glycogen and total lipids (fatty acids plus non-saponifiable material of the liver) were the same as were previously employed (2). Apparent differences were tested for significance by the t method of Fisher (8).

Results

Series A—The liver glycogen and lipid values in the unfasted controls and after 12, 24, and 36 hours of fasting are given in Table I. The difference in glycogen levels in the unfasted animals on the two diets is of the same order as was found previously (2). Statistical evaluation of this difference indicates that the possibility of its being due to chance is 1 in 98. After 12 hours of fasting, the glycogen has dropped 88 per cent in the fatty livers and 54 per cent in the normal livers. The livers of the animals on Diet 2 now contain greater amounts of glycogen than the livers of those on Diet 1. The values for the 24 and 36 hour periods indicate a reduced rate of removal when compared to that during the first 12 hours of fasting. Moreover, the rate of removal during these periods is of the same order of

[2] Generously supplied by the Corn Products Refining Company, New York.

magnitude in both dietary groups. Thus, at the end of 24 and 36 hours of fasting the livers of animals on Diet 2 contain levels of glycogen significantly higher than those of the rats on Diet 1.

The livers of the animals receiving Diet 1 contained the expected high levels of fat, while those on Diet 2 are within the normal range. Statistical analysis of the data shows that there was no significant change in the liver lipids in either dietary group during the 36 hour fast.

TABLE I

Series A, Glycogen and Lipids in Livers of Fasted Male Rats

The animals received the diets for 26 days. The liver data are calculated on the basis of 100 gm. of body weight.

Diet No	No. of rats	Duration of fast	Liver glycogen*	Difference	t calculated	Significant value for t†	Total lipid
		hrs.	*gm.*	*gm.*			*gm.*
1	5	0	0.172 ±0.031				1.031 ±0.211
2	5	0	0.072 ±0.011	0.100	3.02	3.36	0.170 ±0.033
1	7	12	0.020 ±0.002				1.592 ±0.308
2	7	12	0.033 ±0.002	0.013	3.58	3.06	0.156 ±0.010
1	8	24	0.017 ±0.002				1.214 ±0.361
2	7	24	0.029 ±0.001	0.012	4 72	3.01	0.111 ±0.004
1	7	36	0.009 ±0.002				0.752 ±0.220
2	7	36	0.017 ±0.002	0.008	3.12	3.06	0 128 ±0.018

* Including the standard error of the mean calculated as follows:

$$\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$$

† Based on a P value of 0.01 (chances of difference in result being due to errors in sampling 1 in 100).

The animals fasted 24 hours served as controls for the experiments on glucose feeding and are included in Table II, which summarizes the data obtained when glucose was administered to rats on Diets 1 and 2 fasted 24 hours. The administered glucose was completely absorbed in 3 hours in both groups. 3 hours after the administration of 3.5 gm. of glucose per kilo the animals on Diet 2 had a higher liver glycogen than those on Diet 1. The difference after 3 hours was not a carry-over from the control values, for the 0.073 gm. increase in the former is significantly greater than the

0.049 gm. increase in the latter. The animals on Diet 2 maintain their high level through 6 hours. The 12 hour value shows a slight decrease from that of 6 hours and after 24 hours a further decrease has occurred. However, the 24 hour value is significantly higher than the control value. In contrast to those on Diet 2, the rats on Diet 1 show a 42 per cent drop in liver glycogen in the second 3 hours following glucose administration, and in 24

TABLE II

Series A, Glycogen and Lipids in Livers of Glucose-Fed Male Rats

The animals received the diets for 26 days. After a 24 hour fast, glucose (3.5 gm. per kilo) was administered by stomach tube and the animals sacrificed after the time intervals shown in the third column. The liver data are calculated on the basis of 100 gm. of body weight.

Diet No.	No. of rats	Time after glucose	Liver glycogen	Difference	t calculated	Significant value for t	Total lipid
		hrs.	*gm.*	*gm.*			*gm.*
1	8	0	0.017 ±0.002				1.214 ±0.361
2	7	0	0 029 ±0.001	0.012	4.72	3 01	0.111 ±0.004
1	8	3	0.066 ±0.005				1.147 ±0.241
2	8	3	0.102 ±0.004	0.036	3 99	2 98	0.134 ±0.007
1	8	6	0.038 ±0.006				1.643 ±0.300
2	8	6	0.098 ±0.003	0.060	6.75	2.98	0.115 ±0.006
1	8	12	0.027 ±0.004				1 539 ±0 280
2	8	12	0.083 ±0.004	0 056	7.97	2.98	0.118 ±0.004
1	7	24	0 023 ±0.005				0.614 ±0.131
2	7	24	0 053 ±0.005	0.030	3.77	3 06	0.097 ±0.006

hours the level has returned to the control value. Throughout the period under study there were greater amounts of glycogen in the livers of the animals on Diet 2 than in the livers of those on Diet 1.

Series B—The studies on females are summarized in Table III. The unfasted animals on Diets 1 and 2 exhibit the same differences in liver glycogen and lipids as the males. The level of glycogen in both dietary groups is lower than was found in the males. This is probably due to the sex difference

described by Deuel and Davis (1). 1 hour after the administration of three hourly doses of glucose there was no significant difference in the liver glycogen of the females on the two diets. In the animals on Diet 5 the liver lipids were normal and the glycogen was at the same level as was found in the group receiving 5 per cent protein but no choline.

TABLE III

Series B, Glycogen and Lipids in Livers of Unfasted, Fasted, and Glucose-Fed Female Rats

The rats received the diets for 35 days. The glucose-fed animals, after a 24 hour fast, were given three hourly doses of glucose (3.5 gm. per kilo) and sacrificed at the end of the 3rd hour. The liver data are calculated on the basis of 100 gm. of body weight.

Diet No.	No. of rats	Treatment	Liver glycogen	Difference	t calculated	Significant value for t	Total lipid
			gm.	gm.			gm.
1	7	Unfasted	0.111 ±0.012				1.514 ±0.288
2	7	"	0 064 ±0.009	0 047	3 09	3 06	0.225 ±0.028
5	7	"	0.103 ±0.008	0 039*	3.20	3 06	0.145 ±0.006
1	8	Fasted	0.008 ±0 001				1.729 ±0.309
2	8	"	0.010 ±0.002	0.002	1 11	2 98	0.238 ±0.021
1	7	Glucose	0 086 ±0.020				1.376 ±0.240
2	8	"	0.060 ±0.018	0.026	1.03	3.01	0.188 ±0.018

* Difference between Diets 2 and 5.

DISCUSSION

These results offer some explanation for our previous findings. In the animals with fatty livers, the rapid drop in liver glycogen to low levels during a 12 hour fast indicates an increased rate of glycogenolysis. The high fasting blood sugar (2) in these animals may thus be explained as due to the increased rate of glycogenolysis during fasting.

In the rats on Diet 1 the administration of a standard dose of glucose produced an increase in liver glycogen, but the rate of deposition was less than in the animals on Diet 2. From the 3rd through the 24th hour following glucose administration, there was a gradual decrease in glycogen to the control value in the rats with fatty livers. This was in contrast to those on

Diet 2 in which there was a plateau from the 3rd to the 6th hour, followed by a gradual decrease but with the 24 hour level significantly above the control value. The slower rate of glycogenesis in the livers of the rats on Diet 1 is also in accord with our previous findings of a decreased tolerance in these animals.

It is evident from the data on females that the higher level of liver glycogen found in males receiving a diet high in fat and low in protein and lipotropic factors is also present in females ingesting such a diet. Inasmuch as the above relationship has now been found in four experiments, it may be considered an established characteristic of these animals. From a comparison of the animals on Diets 1, 2, and 5 it may be concluded that the high liver fat does not influence the level of liver glycogen. This confirms our previous findings (2). When the females on Diets 1 and 2 were fasted 24 hours and then flooded with glucose for a 3 hour period, there was no difference in the level of the liver glycogen in the two groups. It appears that in the presence of sufficient glucose over a period of time, the animals on Diet 1 can deposit as much glycogen as those on Diet 2. A point requiring further study is the absence of a significant difference between the females on Diets 1 and 2 after a 24 hour fast.

Newburgh and Conn (9) have suggested that an abnormal accumulation of fat in the liver of obese diabetic patients interferes with the liver's capacity to store glycogen at the normal rate. Our findings appear to be in line with this suggestion. However, we are not convinced that the above theory adequately explains the phenomena under study. First, the rapid removal of glycogen from the fatty liver during fasting is difficult to explain simply on the basis of the presence of an abnormal amount of fat in the liver. Secondly, in the animals receiving glucose, after the peak of glycogen deposition was reached there was a relatively rapid removal. This was in contrast to the normal animals in which there was a plateau in the level of liver glycogen followed by a slow rate of removal during the ensuing 18 hours. Thirdly, in the unfasted animals, the glycogen level was not influenced by the amount of fat in the liver. We interpret the data obtained thus far as indicating that the large amounts of lipids in the livers of animals receiving Diet 1 have no influence on the capacity of the liver to metabolize carbohydrates. The observed changes in liver glycogen may reflect an increased demand for glucose by other tissues. Possible causes of an increased demand for carbohydrates are, first, an inadequate supply of "stored" protein for catabolism (10), and, secondly, changes in normal carbohydrate metabolism which are related to the deficient intake of protein and lipotropic factors.

Evidence is being accumulated in this laboratory which indicates that, in animals receiving a diet low in protein and choline, there are fundamental

changes in metabolism which cannot be attributed solely to a lack of lipotropic factors.

SUMMARY

1. In unfasted male and female rats receiving a diet high in fat and low in protein and lipotropic factors, the livers contained significantly greater amounts of glycogen and lipids than the livers of those on a diet high in fat and protein. The level of the liver glycogen was independent of the amount of fat in the liver.

2. The animals having fatty livers exhibited an increased rate of glycogenolysis during fasting and a decreased glycogenesis following a standard dose of glucose.

3. There was no change in the total lipids of the liver during a 36 hour fast.

BIBLIOGRAPHY

1. Deuel, H. J., Jr., and Davis, A., *J. Biol. Chem.*, **146**, 649 (1942).
2. Treadwell, C. R., King, W. C., Bebb, K. C., and Tidwell, H. C., *J. Biol. Chem.*, **143**, 203 (1942).
3. MacLean, D. L., Ridout, J. H., and Best, C. H., *Brit. J. Exp. Path.*, **18**, 345 (1937).
4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
5. Cori, C. F., *J. Biol. Chem.*, **66**, 691 (1925).
6. Deuel, H. J., Jr., Hallman, L. F., Murray, S., and Samuels, L. T., *J. Biol. Chem.*, **119**, 607 (1937).
7. MacKay, E. M., and Clark, W. G., *Am. J. Physiol.*, **135**, 187 (1941-42).
8. Fisher, R. A., Statistical methods for research workers, Edinburgh, 7th edition (1938).
9. Newburgh, L. H., and Conn, J. W., *J. Am. Med. Assn.*, **112**, 7 (1939).
10. MacKay, E. M., Carne, H. O., Wick, A. N., and Visscher, F. E., *J. Biol. Chem.*, **141**, 889 (1941).

THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

II. SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE*

By W. C. SCHNEIDER AND V. R. POTTER

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison)

(Received for publication, April 2, 1943)

Until methods for the assay of animal tissues for specific respiratory enzymes have been developed, the brilliant and fundamental researches of Warburg, Meyerhof, Cori, and others cannot be applied to the various aspects of metabolism. By means of the homogenization technique of Potter and Elvehjem (2), it is possible to disperse cellular components so widely that endogenous respiration is virtually stopped due to dilution effects (2), after which particular phases of metabolism may be restored by the addition of the appropriate substrates and soluble cofactors.[1] The goal is to isolate a particular *reaction* and, by so doing, to determine quantitatively the specific enzyme which catalyzes the reaction, rather than to effect a partial isolation of the enzymes by means of fractionations in which the over-all yield is never determined. In the case of the respiratory enzymes, the situation is complicated by the fact that the reaction occurs through the mediation of a series of enzymes, all of which are necessary to obtain oxygen uptake.

We have previously presented a method for the quantitative determination of cytochrome *c* (1) in animal tissues. It was possible in that instance to express the results on an absolute basis; *i.e.*, in gm. or moles per unit of tissue. In the case of most of the respiratory enzymes, there is no way of expressing the results on an absolute basis, since the measurement is in terms of catalytic effect, and the results must be expressed in the same terms, since no standards are available for comparison. However, by expressing the results in terms of the conventional Q_{O_2}, one may compare the results directly with measurements made on tissue slices. This cannot be done when succinic dehydrogenase is measured by the methylene blue technique (3).

* This work was aided by the Jonathan Bowman Fund for Cancer Research.

The paper on the quantitative determination of cytochrome *c* (1) will be considered as Paper I of this series. Preliminary experiments on coenzyme I-cytochrome reductase as well as the coenzyme I requiring dehydrogenases have been carried out.

[1] The wide-spread application of the homogenization technique without due regard for the nature of the homogenate makes it necessary to emphasize here that in all systems known to involve soluble cofactors the dilution and destruction of these factors must be taken into account.

The present paper involves the assay for two enzymes, succinic dehydrogenase and cytochrome oxidase. They are studied together because it is necessary to prove that when succinate is being oxidized by a tissue homogenate in the presence of excess cytochrome *c*, the limiting factor is succinic dehydrogenase. If this is true, the Q_{O_2} is the measure of succinic dehydrogenase; but it is only true if cytochrome *c* and cytochrome oxidase are present in excess. That cytochrome *c* is present in excess can be assured by preparing the pure compound and adding an amount such that additional cytochrome *c* does not increase the rate of oxygen uptake. In the case of cytochrome oxidase, however, an assay for this enzyme must be devised and applied to every tissue in which succinic dehydrogenase is measured. Although a number of methods for assaying cytochrome oxidase have been published, they have all depended upon the fact that cytochrome *c* can be reduced directly (*i.e.*, non-enzymatically) by certain more or less autoxidizable compounds. The test consists of adding sufficient reducer and cytochrome *c* to keep the oxidase saturated with reduced cytochrome *c*. Previous investigators, in attempts to measure oxidase activity, used hydroquinone (4–6) and *p*-phenylenediamine (6, 7) to reduce cytochrome *c*, while cysteine (6) and ascorbic acid (8) were also indicated as possible reducing agents for cytochrome *c*.

The assay is complicated by the fact that the preparation of the tissue affects the results considerably. Any method of preparing extracts which fails to establish the relation between the tissue and the extract may be eliminated at the start. On the other hand the technique of homogenization has a profound effect upon the results, since we obtained the remarkable result that only those cells which are broken contribute to the rate of oxygen uptake in the cytochrome oxidase system. Since the per cent of whole cells in a homogenate depends upon the homogenizing technique, it is important to be able to control this factor.

EXPERIMENTAL

Materials and Methods

All experimental and assay work was carried out with a conventional Warburg apparatus at 38°.

Tissue Preparation—The tissue was prepared by homogenizing (2) in M/30 phosphate buffer at pH 7.4 or in distilled water.

Cytochrome c—This was prepared from fresh beef hearts as described by Keilin and Hartree (9) except that it was dialyzed against redistilled water instead of 1 per cent NaCl (3).

Substrates—Sodium succinate was prepared as described in a previous report (10). Hydroquinone (Merck's) was purified by sublimation between watch-glasses. Eastman's *d*-isoascorbic acid and Merck's ascorbic

acid and cysteine hydrochloride were used as such without further treatment.

Semicarbazide Hydrochloride—Merck's semicarbazide hydrochloride was recrystallized by dissolving it in the minimal amount of hot water, adding 2 to 3 volumes of ethyl alcohol, and allowing the mixture to stand in the cold until crystallization was complete. The product was washed with alcohol and dried in air.

Metal Ion Solutions—Calcium and aluminum ion solutions were prepared from reagent chemicals at a strength of 4×10^{-3} M.

Whole Cell Preparations—Rat liver was forced through cheese-cloth and then through bolting silk. The resulting mass of tissue was made up to a 1:10 suspension with M/30 phosphate buffer, pH 7.4. The whole cells were separated and washed several times by centrifuging the suspension for 1 minute at 800 R.P.M.

Cell-Free Preparation—A 10 per cent homogenate was centrifuged for 5 minutes at 2000 R.P.M. The residue was made up to the original volume and recentrifuged as before. The combined supernatants were centrifuged under the same conditions to clear out any traces of cells or nuclei.

Succinoxidase System

pH—The effect of pH was determined by using various mixtures of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 in place of the usual phosphate buffer at pH 7.4. The latter served as a control. After the oxygen uptakes were measured, the contents of duplicate flasks were mixed and the pH determined electrometrically. The results of such an experiment are given in Table I; they indicate that the succinoxidase pH optimum range is from 7.5 to 7.7 and that the activity observed with the usual phosphate buffer at pH 7.4 was the same as that given by the phosphate mixtures in the optimum pH range.

Phosphate Concentration—There was little difference in the activity in the range of phosphate concentrations of 0.0201 to 0.0469 M. A slight loss in activity occurred, however, at a phosphate concentration of 0.0067 M. These results are summarized in Table I.

Succinate Concentration—Experiments on succinate concentration by Mr. K. P. DuBois yielded the results given in Table I. The optimum succinate concentration range was from 0.033 to 0.067 M.

Cytochrome c Concentration—The effect of cytochrome *c* concentration on the succinoxidase system was reported previously (3). Those experiments were conducted at 20°, however, and in the absence of calcium and aluminum ions. Changing the temperature to 38° and adding these ions had no effect on the amount of cytochrome *c* needed to saturate the system (see Table I and (3)).

Metal Ion Effects—Although the need for the various components has

been demonstrated previously (3, 10), it is no longer necessary to assume that calcium and aluminum ions are components of the succinoxidase system, since alternate and more likely explanations of their action are now available. Both ions appear to act as specific anti-inhibitors rather than directly as activators of the succinoxidase system. Swingle *et al.* (11) have obtained evidence that calcium activates coenzyme I nucleotidase or some similar enzyme. According to these workers, calcium hastens the complete destruction of the coenzyme I in the liver homogenate. Unless the coenzyme I is completely destroyed, traces of oxalacetate will be

TABLE I

Optimum Conditions for Succinoxidase and Cytochrome Oxidase Systems

Each of the components was varied independently of the other components in the system; *i.e.*, all other components were at optimum levels while one component was varied. The values of the figures given under the various columns are as follows: succinate, M × 10^2; phosphate and ascorbate, M × 10^3; cytochrome *c*, M × 10^5. 10 mg. of fresh liver were used throughout the succinoxidase system. 5 mg. were used in the cytochrome oxidase system except where indicated. Q_{O_2} is the c.mm. of O_2 taken up per hour per mg. of dry tissue.

Succinoxidase system								Cytochrome oxidase system					
Succinate varied	Q_{O_2}	pH varied	Q_{O_2}	Phosphate varied	Q_{O_2}	Cytochrome *c* varied	Q_{O_2}	Cytochrome *c* varied	Q_{O_2}	Ascorbate varied	Q_{O_2}	Fresh liver varied	O_2 per 10 min
												mg.	*c mm.*
0.8	39.0	6.08	23.3	6.7	98	0.00	26.2	0.0	11	3.8	169	0	5 6*
1.7	82.5	6 35	32.8	20.1	107	0.33	66.3	2.4	99	7 6	246	1.0	30 4
3.3	98.5	6.83	42.4	26.8	107	1.00	80.3	4.8	158	11.4	240	1.5	43.2
5.0	98.5	7.34	63.8	33.5	107	1.67	78.6	6.4	170	15.2	253	2.0	56.0
6.7	96.5	7.50†	77.2	40.2	105	2.00	80.8	7.2	184	19.0	228	2.5	67.9
10.0	79.5	7.65	78.5	46.9	105	3.33	80.4	8.0	188	22.8	253		
15.0	52.0	8.32	47.2			10.00	86.0	8.8	190				

* O_2 uptake resulting from extrapolation of other values to zero tissue concentration.

† Control flask containing 1.0 ml. of 0.1 M phosphate buffer, pH 7.4.

formed as soon as succinate is oxidized to fumarate. Only traces of oxalacetate are required to inhibit succinic dehydrogenase. Thus, the calcium affects the reaction between succinate and the dehydrogenase (10) but in a very indirect manner, probably by preventing the formation of oxalacetate. The action of aluminum likewise may be indirect. The apparent dilution effect which was corrected by adding aluminum ions to an otherwise complete system (10) can also be explained in terms of an anti-inhibitor effect on the basis of the following facts. If the observed rate is plotted against the enzyme concentration, a straight line results

which intersects the enzyme scale instead of passing through the origin (Fig. 1, *B*, Ca^{++} curve). It is as if a small amount of heavy metal impurity reacted stoichiometrically with a small portion of the enzyme. The effect is more noticeable with smaller amounts of enzyme[2] and gives the impression of a dilution effect when Q_{O_2} is plotted against enzyme concentration (Fig. 1, *A*, Ca^{++} curve). A true dilution effect will not yield a straight line when the observed rate is plotted against the enzyme concentration (2), as was done in Fig. 1, *B*, Ca^{++} curve. The addition of aluminum appears to prevent the toxic action, and the straight line now intersects the origin (Fig. 1, *B*, $Ca^{++} + Al^{+++}$ curve). It is possible that the in-

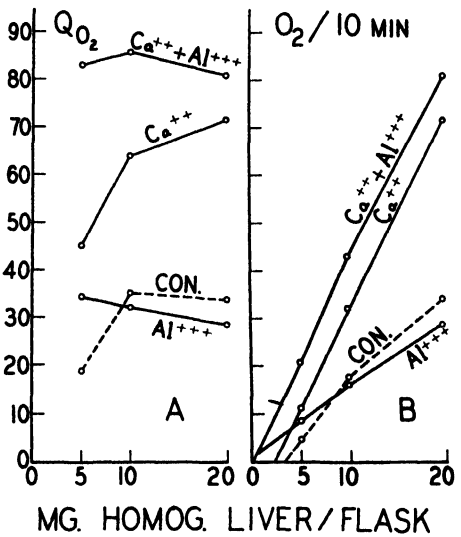


FIG. 1. Ion effects in the succinoxidase system. Curves plotted from data in (10) to show that the lowered activity due to lack of aluminum is not a true dilution effect. See the text. Each point is the average of twelve manometric determinations. Experimental details are given in (10), Table I.

hibitor is a metal ion, since the marked autoxidation of ascorbic acid in phosphate buffer is greatly diminished by adding aluminum ions.

Tissue Concentrations—The dilution effect previously demonstrated in the succinoxidase system (10) was shown to be eliminated by the use of

[2] Axelrod, Swingle, and Elvehjem (12) reported that they did not observe the aluminum effect in their experiments with succinoxidase. They used 40 mg. of tissue per flask, since the larger value for V_G in the Barcroft apparatus requires the use of larger amounts of tissue in order to get manometric displacements of sufficient magnitude. Fig. 1, *A* plainly shows that the aluminum effect is most noticeable at levels too small to be used in their apparatus, while extrapolation of our curve to 40 mg. would result in an absence of aluminum effect, in agreement with their result.

aluminum ions plus calcium ions. That is, under the prescribed conditions, the oxygen uptake is proportional to the tissue concentration. One has only to choose the amount of tissue which will give the most accurately measured rate of oxygen uptake.

Cytochrome Oxidase System

Conditions in the cytochrome oxidase system were kept as nearly like those of the succinoxidase system as possible; that is, the same temperature, phosphate concentration, and pH were used as in the succinoxidase system. However, the cytochrome *c* concentration had to be increased considerably, as will be shown below.

Substrates—Hydroquinone, *p*-phenylenediamine, ascorbic acid, and cysteine were used as substrates for the oxidase. The *p*-phenylenediamine was completely unsatisfactory because the rates of oxygen uptake fell off rapidly. If hydroquinone is used, it is necessary to use semicarbazide in a final concentration of 0.1 M in order to get linear rates of oxygen uptake for at least 20 minutes (*cf.* (5)). When such a system was used, lower rates of oxygen uptake ($Q_{O_2} = 197$) were observed than when ascorbic acid was used as a substrate ($Q_{O_2} = 277$). This lower rate might be ascribed to inhibition by the semicarbazide or to an alternate oxidative pathway in the case of ascorbic acid. That the lower rate is not due to inhibition by the semicarbazide is shown by the fact that identical rates are obtained in the first 5 minutes after hydroquinone has been added in the presence or absence of semicarbazide. From a consideration of oxidation-reduction potentials, an alternate oxidative pathway is unlikely in the case of either hydroquinone or ascorbic acid, whereas such a pathway might easily be involved in the oxidation of cysteine or *p*-phenylenediamine. That any alternate path in the case of the oxidation of ascorbic acid must be very small is indicated by the rate in the absence of added cytochrome *c*, which is about the same as the rate of autoxidation. Cysteine was found to be unsuitable because it is unable to reduce cytochrome *c* rapidly enough to keep the oxidase saturated with reduced cytochrome *c*. Ascorbic acid on the other hand reduced cytochrome *c* rapidly, and gave linear rates of oxygen uptake. In addition it had a low rate of autoxidation and its end-products do not affect the reaction.

The effect of ascorbic acid concentration is given in Table I; there is little difference in the activities in the ascorbate concentration range of 0.0076 to 0.0228 M.

Cytochrome c Concentration—Table I shows the effect of cytochrome *c* concentration on the succinoxidase and the cytochrome oxidase systems. There are two points of interest, (*a*) the oxidase requires much more cytochrome *c* for saturation than does the succinoxidase system, and (*b*)

the oxidase operates at a much slower rate at a cytochrome *c* concentration known to approximate the physiological level in the intact liver cell (3) than does the succinoxidase system. Potter[3] has shown that a rat liver homogenate is not homogeneous in composition and that a partial separation of whole cells from cytoplasm fractions can be effected. Table II shows the results of using partially separated whole cell and cytoplasm preparations in the succinoxidase and the cytochrome oxidase systems. In the absence of added cytochrome *c*, there is a large oxygen uptake in the whole cell preparation with succinate as substrate, while there is practically no uptake with the ascorbate as substrate. This indicates that the succinate diffuses into the cells, whereas the ascorbate does not. This is

TABLE II

Effect of Added Cytochrome c on Succinoxidase (Succinate) and Cytochrome Oxidase (Ascorbate) Activities of Whole Cell and Cell-Free Liver Preparations and on Activities of Liver Homogenized in Isotonic Phosphate and in Water

All ascorbate and succinate values are given as c.mm. of O_2 per 10 minutes.

Added cytochrome *c*	Whole cell preparation		Cell-free preparation		Tissue homogenized in M/30 phosphate		Tissue homogenized in H_2O	
	On succinate	On ascorbate	On succinate	On ascorbate	On succinate	On ascorbate	On succinate	On ascorbate
$M \times 10^5$								
0	49 6	3.7	12.5	5.8	14.0	3.0	9.0	2.6
1.0			31.6	25.7				
2.0	83 5	17.3	42.3	40.6	44.8	28.0	42.5	29.0
3 0			48 2	53.4				
4.0	88.0	25.9	48.5	64.4	45.8	49.4	44.8	59.2
0*					81 0	6.1	38.6	6.1

* 40 mg. of fresh liver were used in this experiment; 10 mg. of fresh liver were used in all the other experiments on tissues homogenized in water and phosphate.

further borne out by the cytoplasm data. In the absence of added cytochrome *c*, the succinate uptake is markedly reduced; this is what one would expect if fewer whole cells were present into which the succinate could diffuse. It also takes much less cytochrome *c* to get the oxidase to operate at a more rapid rate than the succinoxidase system in the cytoplasm preparation than in the whole cell preparation. The question arises as to how one can improve the homogenization technique so as to remove the whole cells more completely. Recent work by Elliott and Libet (13) indicated that this end might be reached by homogenization in distilled water. Table II shows that this procedure certainly produces a marked difference in the succinoxidase activities in the absence of cytochrome *c* (this is espe-

[3] Unpublished experiments.

cially apparent when 40 mg. of tissue were used), indicating that the percentage of cells disrupted in the water treatment is much greater than when the tissue was homogenized in isotonic phosphate. That there is little loss in activity during homogenization in water is shown by the fact that both homogenates have essentially the same succinoxidase activities in the presence of excess added cytochrome *c*. The fact that succinate can diffuse into intact cells suggests a method for determining the proportion of whole cells present. If the succinoxidase activities are determined in the absence of added cytochrome *c* and in the presence of excess added cytochrome *c*, the ratio of these two values should be a measure of the intact cells present, since only the intact cells contribute to the oxygen uptake in the absence of added cytochrome *c*. This was the method which was actually used to correct the observed cytochrome oxidase activities for the degree of homogenization.

Tissue Concentration—That the rate of oxygen uptake is proportional to the tissue concentration is evident from Table I. If, however, the corresponding Q_{O_2} values are plotted against tissue concentration, an inverse dilution effect results (see also the data in (7)); that is, the Q_{O_2} is much higher at lower tissue concentrations than at higher tissue concentrations. If, now, we consider the uptake at zero tissue concentration (obtained by extrapolation of values obtained at three or more tissue concentrations) to be a measure of the ascorbate autoxidation and subtract this value from the uptake at all tissue levels, the Q_{O_2} values are constant and independent of tissue concentration. This procedure was employed in all assay work and proved highly successful. Q_{O_2} values determined in this way represent at least minimal values for the cytochrome oxidase system. It was also observed that apparent dilution effects sometimes resulted when extremely small amounts of homogenate were used, as in the case of the more active tissues, and that these effects could be corrected by the addition of $AlCl_3$ just as in the succinoxidase system. The mechanism of action is probably also the same.

Assay Methods and Results

In the assay of various tissues for succinoxidase and cytochrome oxidase activities, two levels of tissue were used in the former and three in the latter. The autoxidation rate for the ascorbic acid was determined indirectly as described above. Experience showed that it was unnecessary to measure the oxygen uptake in the absence of succinate or ascorbate, since this was always found to be insignificant. Succinoxidase rates and corrected cytochrome oxidase rates were then converted to Q_{O_2} values and the values for the latter assay were also corrected for the degree of homogeniza-

tion of the tissue. The activities were determined simultaneously on each sample. A summary of the assay conditions used was as follows:

Succinoxidase System—1.0 ml. of 0.1 M phosphate buffer (pH 7.4), 0.4 ml. of 10^{-4} M cytochrome *c*, 0.3 ml. of 4×10^{-3} M $CaCl_2$, 0.3 ml. of 4×10^{-3} M $AlCl_3$, 0.3 ml. of 0.5 M sodium succinate (pH 7.4), the desired amount of tissue, and water to make 3.0 ml. were used; 0.2 ml. of 2 N NaOH was placed in the center cup. Two levels of tissue were assayed, with the higher level also tested in the absence of added cytochrome *c*.

Cytochrome Oxidase System—1.0 ml. of 0.1 M phosphate buffer (pH 7.4), 1.0 ml. of 2.4×10^{-4} M cytochrome *c*, 0.3 ml. of 0.114 M ascorbic acid neutralized to pH 7.4 with NaOH, 0.3 ml. of 4×10^{-3} M $AlCl_3$, the desired amount of tissue, and water to make 3.0 ml. were used; 0.2 ml. of 2 N NaOH was placed in the center cup. Three levels of tissue were tested.

Glass-redistilled water was used throughout in both systems. It was possible with the technique described to assay for both enzymes with six manometers per tissue sample.

A large variety of tissues has been assayed for succinic dehydrogenase and cytochrome oxidase activity. These results were reported in detail elsewhere in connection with a survey of cancer tissues (14). A summary of these results as well as those of other authors is presented in Table III for purposes of comparison. It is evident that our Q_{O_2} values are considerably higher than those of previous workers, with the exception of those of Axelrod *et al.* This similarity in results is difficult to explain in the case of the values with cytochrome oxidase, since these authors used hydroquinone as a substrate and we consistently found that hydroquinone gave lower activities than did ascorbic acid. A possible explanation may lie in the nutritional state of the two groups of animals or in the age of the animals, since we have been able to show that the cytochrome oxidase activity is considerably higher in young rats than it is in adult rats (unpublished experiments).

The succinoxidase test system as described in this paper has already been widely applied in other biological studies. Among these might be mentioned the study of the succinoxidase system in riboflavin-deficient rats (12, 16) and the effect of inhibitors on the succinoxidase system (17, 18). A recent study (19) in which this technique was used showed that the succinoxidase activity was considerably increased in the muscles of vitamin E-deficient animals, and that this enhanced activity could be reduced to the normal range by the addition of α-tocopherol phosphate to the system. The demonstration of this effect of vitamin E on the succinoxidase system poses the question as to whether the activities as measured in normal tissues are limited by the vitamin E content of the tissues studied,

but on the basis of calculations on the tocopherol content of cattle muscle (20) it seems likely that the concentrations used by Houchin (19) to obtain

TABLE III

Succinoxidase and Cytochrome Oxidase Activities of Rat Tissues

All results are reported as Q_{O_2} values; *i.e.*, as c.mm. of O_2 taken up per hour per mg. of dry tissue.

Tissue	Succinoxidase Q_{O_2}			Cytochrome oxidase Q_{O_2}						
		From (7)*	From (12)†		‡	From (7)*	From (12)†	From (4)§	From (5)||	From (15)¶
Heart	219 (197–250)	62		974 (693–1170)	1699 (1405–1870)	506		97	255	299
Kidney	195 (174–226)	112		549 (511–585)	695 (663–725)	288		47	146	
Liver	87.7 (76.8–101)	66	84 (78–98)	392 (281–518)	479 (347–586)	167	434 (348–480)	17		107
Brain	48.7 (41–64.1)	18		420 (306–584)	476 (348–654)	134		35		
Skeletal muscle	35.5 (29.2–48.7)	6.6		180 (110–274)	271 (171–372)	38		23		35
Spleen	23 3 (19.4–35.3)	0.5		195 (158–317)	222 (178–374)	32		16		46.2
Lung	17.9 (15.0–21.6)	7.5		92.3 (70.6–114)	109 (83.0–129)	31		13		
Jensen sarcoma	17.8 (15.3–21.1)	13		129 (106–170)	145 (121–192)	43				10.2

* Succinate and *p*-phenylenediamine substrates; homogenization. The figures in parentheses in the headings refer to the bibliography.

† Succinate and hydroquinone substrates; homogenization. Semicarbazide quinone fixation.

‡ The results in this column were obtained by correcting for the degree of homogenization of the tissues.

§ Hydroquinone substrate; extraction by dialysis for 10 to 15 hours.

|| Hydroquinone substrate; homogenization. Semicarbazide quinone fixation.

¶ *p*-Phenylenediamine substrate; homogenization and extraction. These results were converted to Q_{O_2} values by using our dry weight determinations.

an effect on the succinoxidase system were considerably above those which would occur in the succinoxidase assay.

SUMMARY

1. The optimum succinate, cytochrome *c*, phosphate, and hydrogen ion concentrations for the succinoxidase system and the optimum ascorbic acid and cytochrome *c* concentrations for the cytochrome oxidase system are reported.

2. Calcium and aluminum ions are necessary for maximum activity in the succinoxidase system; aluminum ions were found to be necessary for the cytochrome oxidase system. The action in both cases is probably indirect.

3. The cytochrome oxidase assay was shown to be limited by the completeness of the disruption of the tissue cells; a method is described for the correction of the observed cytochrome oxidase activity for the degree of homogenization of the tissue.

4. A method is described for correction of the cytochrome oxidase oxygen uptake rates for the autoxidation of the ascorbic acid.

5. The heart, kidney, liver, brain, skeletal muscle, spleen, and lungs of the rat were assayed for succinic dehydrogenase and cytochrome oxidase activities.

BIBLIOGRAPHY

1. Potter, V. R., and DuBois, K. P., *J. Biol. Chem.*, **142**, 417 (1942).
2. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
3. Potter, V. R., *J. Biol. Chem.*, **141**, 775 (1941).
4. Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
5. Schultze, M. O., *J. Biol. Chem.*, **129**, 729 (1939).
6. Krahl, M. E., Keltch, A. K., Neubeck, C. E., and Clowes, G. H. A., *J. Gen. Physiol.*, **24**, 597 (1941).
7. Elliott, K. A., and Greig, M. E., *Biochem. J.*, **32**, 1407 (1938).
8. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **125**, 171 (1938).
9. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
10. Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, **142**, 543 (1942).
11. Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 581 (1942).
12. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 297 (1942).
13. Elliott, K. A. C., and Libet, B., *J. Biol. Chem.*, **143**, 227 (1942).
14. Schneider, W. C., and Potter, V. R., *Cancer Research*, in press.
15. Shack, J., *J. Nat. Cancer Inst.*, **3**, 389 (1943).
16. Axelrod, A. E., Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **142**, 85 (1942).
17. Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, **26**, 391 (1943).
18. Potter, V. R., and Albaum, H. G., *J. Gen. Physiol.*, in press.
19. Houchin, O. B., *J. Biol. Chem.*, **146**, 313 (1942).
20. Karrer, P., Jaeger, W., and Keller, H., *Helv. chim. acta*, **23**, 464 (1940).

THE RELATIONSHIP OF ACETOIN TO METABOLIC ACETYLATIONS

By E. A. DOISY, Jr., and W. W. WESTERFELD

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

(Received for publication, April 26, 1943)

The metabolic acetylation of amino groups is a well known "detoxification" reaction (1, 2). Aromatic amines, such as *p*-aminobenzoic acid (3, 4) and sulfanilamide (5), are readily acetylated by rabbits and humans, while the acetylation of the aliphatic amino group in phenylaminobutyric acid has been observed in dogs (6) and rats (7). The amino group of cysteine is acetylated in the synthesis of mercapturic acids (8). Metabolic acetylations are not confined to amino groups, since the formation of acetylcholine is constantly taking place in nervous tissue (9–11).

The nature of the acetylating agent, *e.g.* the substance furnishing the acetyl group for these reactions, has not been established, but it has often been assumed to be acetate (12, 13). Studies of the effect of acetate administration on the acetylation reaction have led to conflicting results (13, 14). Other studies have indicated that the acetylating agent is derived from carbohydrate (11, 15, 16), and that pyruvate is a probable precursor (11, 12, 17). Furthermore, it has been shown that the acetylation of choline is dependent upon an adequate supply of thiamine (18). In view of the fact that pyruvate is converted to acetoin by a diphosphothiamine enzyme in mammalian tissues (19), it seemed likely that acetoin also would be a precursor of the acetylating agent.

Experimental studies have shown that acetoin and the related 2,3-butylene glycol increased the acetylation of *p*-aminobenzoic acid by rabbits. Acetate had little or no effect upon this reaction, and it is therefore unlikely that the effect of acetoin and related compounds could be due to conversion to acetate. The most likely chain of events is represented in Fig. 1, in which acetyl phosphate is believed to be the acetylating agent.

EXPERIMENTAL

Male rabbits weighing about 2 kilos were placed in metabolism cages and fed a diet of rabbit chow *ad libitum*. At intervals of once or twice per week, each rabbit was injected subcutaneously with 1 gm. of *p*-aminobenzoic acid as the sodium salt in 10 per cent solution. Urine was collected daily for 48 hours under a small amount of toluene, and the total urine sample was diluted to 2 liters. An aliquot was further diluted 1:100; 30 cc. of this diluted solution were mixed with 5 cc. of 4 N HCl and diluted to 100 cc.

Free *p*-aminobenzoic acid was determined colorimetrically on 10 cc. aliquots of this solution. A 20 cc. aliquot was heated in boiling water for 1 hour, cooled, diluted to 25 cc., and the total *p*-aminobenzoic acid determined colorimetrically on 10 cc. aliquots. The difference between free and total *p*-aminobenzoic acid represented the acetylated form. The assumption that essentially all of this difference is due to acetylated *p*-aminobenzoic acid seems justified, since (*a*) this difference is actually a measure of the bound amino groups, and acetylation is the only known metabolic mechanism for blocking the amino group in this compound, and (*b*) the amounts of acetylated *p*-aminobenzoic acid previously isolated in similar experiments (15) are of the same magnitude as those found by colorimetric analysis.

When repeated tests showed that a rabbit was acetylating a relatively constant percentage of the *p*-aminobenzoic acid, the effect of the administration of various substances upon this acetylation reaction was studied. The test substance was injected subcutaneously as a 10 or 20 per cent solution in three equal portions, the first one simultaneously with the 1 gm. of

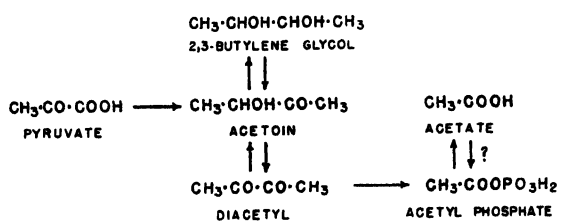


FIG. 1. Formation of the acetylating agent from carbohydrate

p-aminobenzoic acid (but at a different site), and the other two following at intervals of 3 hours. Any change in the amount of *p*-aminobenzoic acid acetylated was attributed to the substance tested, provided this change was sufficiently large. This general procedure for studying the acetylation reaction has been widely used (15–17, 20).

Determination of p-Aminobenzoic Acid—*p*-Aminobenzoic acid was determined by the colorimetric method developed for sulfanilamide by Bratton and Marshall (21). The diazotization of *p*-aminobenzoic acid and coupling with N-(1-naphthyl)ethylenediamine were carried out with the reagents and in the manner described for sulfanilamide. The purple-red azo dye so produced was read in an Evelyn photoelectric colorimeter with a 540 mμ filter. A 0.1 mg. per cent solution of *p*-aminobenzoic acid gave about 30 per cent as much transmission of light as a blank composed of the reagents alone. When log I_0/I was plotted against concentration, a straight line was obtained.

A 3-fold increase in the time allowed for the various reactions to take place had no effect on the amount of color obtained. 5 minutes were al-

lowed for full development of the color after the addition of N-(1-naphthyl)-ethylenediamine, and the color was then stable for at least 1 hour. Carrying out the reaction at 16° instead of room temperature (26°) gave incomplete color development. To avoid errors due to possible fluctuations in temperature or unknown factors, a 0.1 mg. per cent standard of *p*-aminobenzoic acid was run simultaneously with all determinations. The acetylated *p*-aminobenzoic acid gave no color in the reaction and had no effect on the development of color with free *p*-aminobenzoic acid. The presence of rabbit urine in an amount equivalent to that obtained in the experimental runs, *i.e.* 1 gm. of *p*-aminobenzoic acid in 200 to 400 cc. of urine, had no effect on the determination.

Determination of Acetylated p-Aminobenzoic Acid—Acetylated *p*-aminobenzoic acid was determined as the difference between the free form and the total *p*-aminobenzoic acid found after acid hydrolysis. Hydrolysis was effected by placing the acidified solution (5 cc. of 4 N HCl in 100 cc. of solution) in boiling water for 1 hour. Although these appeared to be the optimum conditions for hydrolysis, incomplete recoveries were usually obtained. Losses appeared to be due primarily to destruction of free *p*-aminobenzoic acid.

Hydrolysis controls were run under the conditions encountered in the analysis of the experimental rabbit urines. The amounts of free and acetylated *p*-aminobenzoic acid were varied, the total remaining fairly constant, and the proper amount of rabbit urine was added. The results are shown in Table I. Under these conditions, the loss by hydrolysis remained fairly constant at 6 per cent of the total *p*-aminobenzoic acid. The values obtained on hydrolysis of the experimental urines were therefore divided by the factor 0.94 to obtain the correct amount of total *p*-aminobenzoic acid present.

Results

Complete protocols for some of the tests on acetoin and 2,3-butylene glycol are recorded in Table II in order to show the method of testing and the variations encountered. Considerable variation in the amount of *p*-aminobenzoic acid acetylated was often encountered in the first few injections, but thereafter the rabbits acetylated a relatively constant percentage of the *p*-aminobenzoic acid. The results were discarded if a consistent base-line could not be established, or if the base-line changed appreciably after any test.

The interval between injections had no apparent effect on the percentage of *p*-aminobenzoic acid acetylated. Rabbit 15 acetylated 37 to 42 per cent of the *p*-aminobenzoic acid for the five control tests preceding the testing of 6 gm. of 2,3-butylene glycol and 36 to 37 per cent for the two controls fol-

lowing; during the glycol administration, the acetylation rose to 55 per cent. Rabbit 17 acetylated 26 to 32 per cent for four control tests preceding the injection, 43 per cent during the injection of 3 gm. of 2,3-butylene glycol, and 34 to 35 per cent after the injection. Rabbit 11 showed somewhat more variation, but the responses to 2,3-butylene glycol and the second acetoin administration were considered positive. The response by Rabbit 13 to the acetoin administration was not sufficiently large to be considered significant.

The remainder of the acetoin tests, as well as the results obtained from the administration of sodium acetate, is summarized in Table III. Rabbits

TABLE I

Loss of p-Aminobenzoic Acid (p-ABA) on Hydrolysis of Mixtures of Free and Acetylated p-Aminobenzoic Acid in Rabbit Urine

The solution hydrolyzed consisted of the indicated amounts of free and acetylated *p*-aminobenzoic acid, 1 cc. of 4 N HCl, 0.6 cc. of 1:100 rabbit urine, and water to a total volume of 20 cc. After heating in boiling water for 1 hour, the solution was diluted to 25 cc. and analyzed.

All concentrations are recorded in mg. per cent of free *p*-aminobenzoic acid in the final 25 cc. volume.

p-ABA added			Total *p* ABA recovered	*p*-ABA loss, per cent of total
Free	Acetyl	Total		
0.0952	0.0122	0.1074	0.1002	6.7
0.084	0.0245	0.1085	0.1020	6.0
0.0728	0.0306	0.1034	0.0965	6.65
0.0728	0.0367	0.1095	0.1025	6.4
0.0616	0.0429	0.1045	0.1002	4.1
0.0616	0.0490	0.1106	0.1040	6.0
0.0504	0.0551	0.1055	0.1000	5.2
0.0504	0.0612	0.1116	0.1055	5.5
Average.....				6.0

2, 3, and 4 received a total of 3, 4.5, and 6 gm. of acetoin, and 3, 4, and 3 gm. of sodium acetate respectively. In each of these tests, acetoin gave significant increases above the control levels in the percentage of *p*-aminobenzoic acid acetylated, whereas the acetate administration either had no effect or decreased the acetylation. Similar results were observed in three of the four remaining acetate tests, in which 6 gm. of sodium acetate were administered to Rabbits 5, 7, and 8, and 3 gm. to Rabbit 6. The one positive response for acetate (Rabbit 7) seemed to be genuine and was duplicated a second time on the same animal.

The results obtained on glucose administration are given in detail in

TABLE II

Effect of Acetoin and 2,3-Butylene Glycol Administration on Acetylation of p-Aminobenzoic Acid by Rabbits

Rabbit No.	Weight	Interval between tests	Substance administered	*p*-ABA excreted		*p*-ABA acetylated
				Free	Total	
	kg.	*days*		*mg.*	*mg.*	*per cent*
15	2.4	Jan. 3, 1943		165	340	52
		20		588	900	35
		5		695	1141	39
		9		523	875	40
		6		561	946	41
		6		566	970	42
		2		618	974	37
		3	6 gm. 2,3-butylene glycol	517	1140	55
		2		667	1044	36
		7		644	1030	37
17	2.3	Jan. 3, 1943		547	887	38
		20		665	950	30
		5		528	887	41
		9		602	875	31
		6		600	806	26
		6		603	891	32
		2		722	974	26
		3	3 gm. 2,3-butylene glycol	586	1022	43
		2		649	981	34
		7		634	973	35
11	2.0	Sept. 6, 1942		346	956	64
		4		826	989	16
		3		863	1033	16
		5		960	1137	16
		6	3 gm. 2,3-butylene glycol	706	949	26
		7		729	853	15
		7		857	1078	21
		3		812	990	18
		11		800	1018	21
		12		798	1043	24
		2	3 gm. acetoin	616	831	26
		7	6 " "	628	901	30
		3		598	740	19
		4		681	823	17
		5		806	1026	21
13	3.3	Oct. 22, 1942		805	918	12
		12		923	1062	13
		2	3 gm. acetoin	792	935	15
		7	6 " "	1050	1248	16
		3		770	899	14
		4		941	1034	9
		5		772	852	9

Table IV in order to show the nature of the response. Rabbits 7 and 9 each received a total of 6 gm. of glucose. An increased acetylation was observed during the administration of glucose, but the maximum effect was obtained several days later; a progressive fall to the starting values took place during the following 2 weeks. Further tests with other substances were discontinued when it appeared that atypical responses were being obtained.

TABLE III

Effect of Acetate and Acetoin Administration on Acetylation of p-Aminobenzoic Acid by Rabbits

Rabbit No. / Weight, *kg.*	2 / 2.3	3 / 1.7	4 / 2.0	5 / 2.3	6 / 1.7	8 / 2.2	7 / 2.0
	Per cent *p*-ABA acetylated						
Control	28	30	41	24	41	55	53
Acetate	28	23	34	21	33	54	60
Control	22	29	43	17			49
Acetoin	35	37	52				

TABLE IV

Effect of Glucose Administration on Acetylation of p-Aminobenzoic Acid by Rabbits

Interval between tests	Substance administered	Rabbit 7, 2.0 kilos			Rabbit 9, 2.0 kilos		
		p-ABA excreted		*p*-ABA acetylated	*p*-ABA excreted		*p*-ABA acetylated
		Free	Total		Free	Total	
days		*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Aug. 27, 1942		490	905	46	667	962	31
3		522	919	43	602	928	35
4	Glucose	365	853	57	522	943	45
3		346	956	64	477	926	49
4		372	922	60	596	1020	42
3		493	955	48	610	1070	43
4		543	1034	47	670	1111	40

DISCUSSION

The demonstration of a relationship between acetoin and the acetylating reaction is a further clarification of the metabolic pathway by which the acetylating agent is formed from carbohydrate. The individual steps from glycogen to pyruvate to acetoin have been defined, and the acetylating agent is apparently formed somewhere between acetoin and acetate in the metabolic pathway. Though direct evidence is lacking, the most probable substance that could fulfil the rôle of an acetylating agent is acetyl phosphate (22, 23).

The relationship of acetate to the acetylation reaction is confusing. It had a stimulating effect on the acetylation of sulfanilamide by liver slices (12), but had no effect on the formation of acetylcholine *in vitro* (11). Some authors (15, 20, 24) have reported it to be effective *in vivo* in studies similar to those reported herein; others (17, 13) have found no effect from acetate administration. When deutero acetic acid was administered simultaneously with sulfanilamide or *p*-aminobenzoic acid, Bernhard (13) recovered a small part of the labeled acetate in the acetylated derivative; Erlenmeyer *et al.* (14) found none of the deutero acetic acid incorporated in the acetyl derivative. On the basis of the energy relationships involved, it is highly improbable that acetate itself could act as an acetylating agent. Such energy is usually supplied in biological reactions by the formation of an energy-rich phosphate bond (23).

The increased acetylation resulting from insulin treatment (15, 16) could be attributed to increased pyruvate formation (25). Previously, the effect of insulin on alcohol metabolism was shown to be a pyruvate effect (26). A splitting of acetoacetate to acetyl phosphate would explain the acetylation effects observed with acetoacetate (12, 20, 24), and would provide a means of forming the acetylating agent from fat.

SUMMARY

The colorimetric method developed by Bratton and Marshall for sulfanilamide was utilized in the determination of *p*-aminobenzoic acid.

The acetylation of *p*-aminobenzoic acid by rabbits was significantly increased by the administration of acetoin and 2,3-butylene glycol; sodium acetate either had no effect or decreased the acetylation in six out of seven rabbits tested.

Glucose administration increased the acetylation, but the maximum effect was delayed several days, and the return to the starting level occurred over a period of 2 weeks.

We are indebted to Dr. F. Visser't Hooft of the Lucidol Corporation, Buffalo, for the acetoin used in these studies.

BIBLIOGRAPHY

1. Sherwin, C. P., *Physiol. Rev.*, **2**, 238 (1922).
2. Young, L., *Physiol. Rev.*, **19**, 323 (1939).
3. Ellinger, A., and Hensel, M., *Z. physiol. Chem.*, **91**, 21 (1914).
4. Muenzen, J. B., Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, **67**, 469 (1926).
5. Marshall, E. K., Jr., Cutting, W. C., and Emerson, K., Jr., *Science*, **85**, 202 (1937).
6. Knoop, F., and Kertes, E., *Z. physiol. Chem.*, **71**, 252 (1911).
7. du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131**, 273 (1939).

8. Stekol, J. A., *J. Biol. Chem.*, **124**, 129 (1938).
9. Stedman, E., and Stedman, E., *Biochem. J.*, **31**, 817 (1937).
10. Quastel, J. H., Tennebaum, M., and Wheatley, A. H. M., *Biochem. J.*, **30**, 1668 (1936).
11. Mann, P. J. G., Tennebaum, M., and Quastel, J. H., *Biochem. J.*, **32**, 243 (1938); **33**, 822, 1506 (1939).
12. Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, **124**, 613 (1938).
13. Bernhard, K., *Z. physiol. Chem.*, **267**, 91, 99 (1940).
14. Erlenmeyer, H., Sullmann, H., and Schenkel, H., *Helv. chim. acta*, **21**, 401 (1938).
15. Harrow, B., Mazur, A., Borek, E., and Sherwin, C. P., *Biochem. Z.*, **293**, 302 (1937).
16. Harrow, B., Mazur, A., and Sherwin, C. P., *J. Biol. Chem.*, **102**, 35 (1933).
17. Knoop, F., *Biochem. Z.*, **127**, 200 (1922).
18. Mann, P. J. G., and Quastel, J. H., *Nature*, **145**, 856 (1940).
19. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69 (1942).
20. Hensel, M., *Z. physiol. Chem.*, **93**, 401 (1915).
21. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).
22. Lipmann, F., *J. Biol. Chem.*, **134**, 463 (1940).
23. Lipmann, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1**, 99 (1941).
24. Harrow, B., Power, F. W., and Sherwin, C. P., *Proc. Soc. Exp. Biol. and Med.*, **24**, 422 (1926).
25. Bueding, E., Fazekas, J. F., Herrlich, H., and Himwich, H. E., *Science*, **95**, 282 (1942).
26. Westerfeld, W. W., Stotz, E., and Berg, R. L., *J. Biol. Chem.*, **144**, 657 (1942).

THE COUPLED OXIDATION-REDUCTION OF ALCOHOL AND PYRUVATE IN VIVO

By W. W. WESTERFELD, ELMER STOTZ, and ROBERT L. BERG

(From the Department of Biological Chemistry, Harvard Medical School, Boston, and the Biochemical Laboratory of the McLean Hospital, Waverley, Massachusetts)

(Received for publication, April 26, 1943)

The coupled oxidation-reduction of alcohol and pyruvate is a coenzyme-linked reaction in which both the oxidation of alcohol (1, 2) and reduction of pyruvate (3) are dependent upon diphosphopyridine nucleotide (cozymase or coenzyme I). In the presence of both substrates, their respective enzymes, and diphosphopyridine nucleotide, hydrogen is transferred from the alcohol to the pyruvate by means of the coenzyme until equilibrium is established. Under conditions which prevent the accumulation of end-products, the net result of the reaction is an oxidation of the alcohol to acetaldehyde and a reduction of the pyruvate to lactate according to the equation,

$$CH_3 \cdot CH_2 \cdot OH + CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CHO + CH_3 \cdot CHOH \cdot COOH$$

Although this reaction has been demonstrated with liver slices *in vitro* by Leloir and Muñoz (4), it has never been established that this or similar coupled reactions actually occur in the intact organism.

A recent investigation (5) showed that the administration of pyruvate accelerated the rate of alcohol metabolism. At that time, it was suggested that this pyruvate effect might be due to a condensation of the pyruvate with acetaldehyde (arising from oxidation of the alcohol) to give acetoin. It was also recognized that the pyruvate effect could be due to a coupled oxidation-reduction of the alcohol and pyruvate.

The development of a satisfactory method for the estimation of small amounts of acetaldehyde in blood (6) provided the means for distinguishing between these two possible explanations, and the experiments herein reported clearly indicate that pyruvate increases the rate of alcohol metabolism principally because it enters into a coupled oxidation-reduction reaction with the alcohol. This allows the alcohol to be oxidized to acetaldehyde more rapidly than is apparently accomplished by direct reaction with oxygen.

EXPERIMENTAL

Blood Alcohol-Acetaldehyde Relations—A fasted dog was given approximately 2 cc. of alcohol per kilo in 20 per cent solution by stomach tube, and

a 5 hour interval was then allowed for complete distribution of the alcohol and the establishment of a constant rate of oxidation. Acetaldehyde levels were determined (6) in blood samples taken from the jugular vein during this control period and immediately after the intravenous injection of 2.5 gm. of sodium pyruvate.

Following the ingestion of alcohol, the blood acetaldehyde increased from the trace normally present to the low value of about 0.05 mg. per cent, and it remained at approximately this level even when the blood alcohol concentration rose to 185 mg. per cent. The changes that followed the intravenous injection of sodium pyruvate are shown in Fig. 1. The administration of pyruvate during alcohol metabolism caused a brief fall in the blood acetaldehyde level, possibly indicating a more rapid removal of the

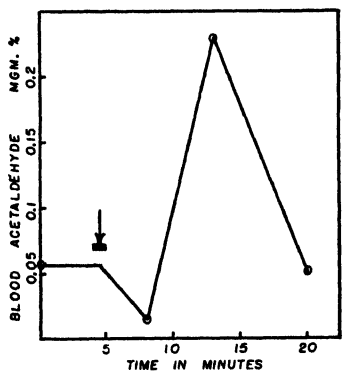


FIG. 1. The effect of sodium pyruvate administration on the blood acetaldehyde concentration during alcohol metabolism. Dog 10M, 14.6 kilos, received 30 cc. of 95 per cent alcohol orally 5 hours earlier. The blood alcohol concentration at 0 time on the graph was 75 mg. per cent. 25 cc. of 10 per cent sodium pyruvate were injected intravenously, as indicated by the arrow.

acetaldehyde, but this was soon followed by a much more pronounced increase. The rise cannot be attributed to the formation of acetaldehyde from the pyruvate administered, since much larger amounts of pyruvate were given to a dog that had received no alcohol without the appearance of any acetaldehyde in the blood.

From a consideration of the increased blood acetaldehyde, together with the data previously published (5), it is apparent that the administration of pyruvate during alcohol metabolism caused an alteration in the metabolic relationships normally existing between pyruvate-lactate and alcohol-acetaldehyde; lactate and acetaldehyde were relatively increased at the expense of alcohol and pyruvate. These changes in all four components of the reaction, and especially the changes in opposite directions between

pyruvate and lactate and between alcohol and acetaldehyde, show that a dismutation reaction has taken place.

Limiting Reaction in Alcohol Metabolism—It is axiomatic that, if pyruvate increases the rate of alcohol metabolism, it must do so by affecting that reaction which is normally the slowest one in the metabolic chain of equilibria reactions. If the rate of alcohol metabolism were normally limited by the maximum rate at which the alcohol could be oxidized to acetaldehyde, then pyruvate must affect this oxidation. Or if the rate were limited by the animal's ability to remove acetaldehyde, then the increased rate obtained with pyruvate must be due to some effect of the pyruvate on the rate of acetaldehyde metabolism. An experiment was, therefore, devised to de-

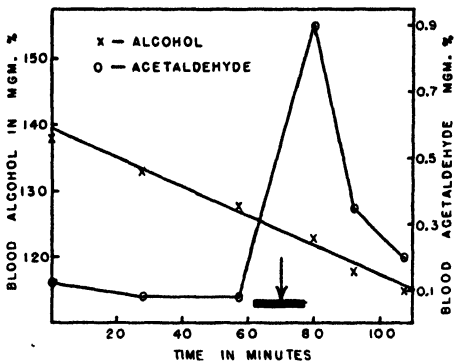


FIG. 2. The metabolic removal of acetaldehyde injected intravenously during alcohol metabolism. Dog 13M, 14.6 kilos, received 35 cc. of 95 per cent alcohol orally 4 hours earlier. The rate of decrease of blood alcohol = 13.2 mg. per cent per hour. 50 cc. of 4 per cent acetaldehyde were injected intravenously, as indicated by the arrow.

termine whether the oxidation of alcohol or the metabolism of acetaldehyde is normally the limiting reaction in alcohol metabolism.

A dog was fed 2.33 cc. of alcohol per kilo and, after a 4 hour interval, the rates of decrease of blood alcohol (5) and acetaldehyde were established. 2 gm. of acetaldehyde were then injected intravenously, with the results illustrated in Fig. 2. The amount of acetaldehyde administered was sufficient to raise the blood level at least 15 mg. per cent if it were completely distributed throughout the body unaltered. Yet 3 minutes after the injection was completed, the blood level was less than 1 mg. per cent, and it had practically returned to the starting value within 30 minutes; the acetaldehyde was not reduced to alcohol, since the blood alcohol curve was unaltered. The essential point of the experiment is that this dog was able to metabolize not only the acetaldehyde produced from the oxidation of the alcohol but an additional 2 gm. superimposed upon it by injection without

affecting the established rate of alcohol metabolism. It proves quite clearly that a dog is able to metabolize acetaldehyde much more rapidly than it is being formed from the alcohol. The slowest reaction in alcohol metabolism, therefore, must be the oxidation of the alcohol itself to acetaldehyde, and the pyruvate effect must be due to increasing the rate of this oxidation. The only possible mechanism known at present whereby this could be accomplished is through a coupled oxidation-reduction of the pyruvate and the alcohol.

It is doubtful that a true equilibrium between alcohol and acetaldehyde exists in dogs, and this species apparently removes acetaldehyde about as rapidly as it can be formed from the alcohol. Blood acetaldehyde levels

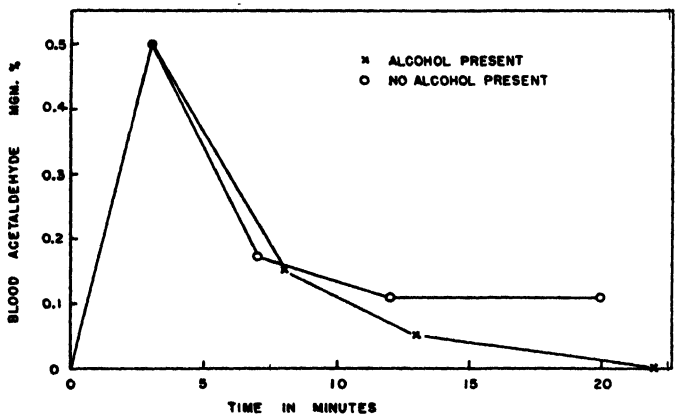


FIG. 3. The metabolic removal of acetaldehyde injected intravenously during and in the absence of alcohol metabolism. Dog 10M, 14.6 kilos, was given 25 cc. of 4 per cent acetaldehyde intravenously during 6 to 7 minutes immediately preceding 0 time. ○ indicates no alcohol administered; ×, 30 cc. of 95 per cent alcohol were given orally 5 hours prior to the acetaldehyde injection. The blood alcohol concentration at 0 time = 72 mg. per cent.

did not parallel the alcohol concentration, and the elevation of the blood acetaldehyde by injection had no effect on the rate of alcohol oxidation.

Acetaldehyde appeared to be very irritating by any route of administration. When 5 gm. of acetaldehyde in 200 cc. of water were fed by stomach tube to 20 kilo dogs, no acetaldehyde or alcohol could be detected in any of the blood samples taken 8 to 80 minutes afterward and the dogs vomited within that period. When a 4 per cent solution of freshly redistilled acetaldehyde in 0.9 per cent saline was injected intravenously, no alcohol was detected in the blood. The rate of acetaldehyde disappearance from the blood was not appreciably different whether or not the dog was metabolizing alcohol at the time of its administration (Fig. 3). Only negligible quanti-

ties of administered acetaldehyde appear to be excreted in the expired air and urine (7).

Effect of Lactate on Blood Alcohol Curve—The effect of lactate administration on the alcohol curve is illustrated in Fig. 4. The experiments were conducted by the methods previously used in determining the pyruvate effect, and the curves illustrated in Fig. 4 were obtained upon the same dogs for which the pyruvate and alanine effects were previously published (5). The effect of lactate in different dogs was variable, but in no case did lactate

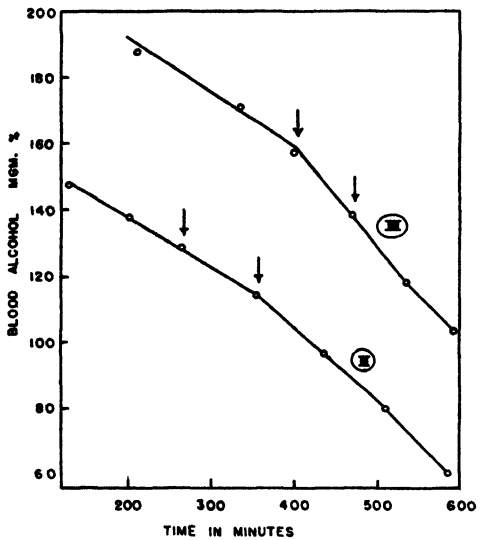


FIG. 4. The effect of *dl*-lactate on the disappearance of blood alcohol. Alcohol was administered in 20 per cent solution by stomach tube at 0 time. 10 gm. of sodium lactate in 125 cc. of water were given orally at the times indicated by the arrows. Curve II, Dog 2F, 9.8 kilos, received 25 cc. of alcohol. The rates of fall of blood alcohol were as follows: control = 9.0 mg. per cent per hour; after lactate, 1st hour = 9.0; 2nd and 3rd hour = 13.2; 4th hour = 16. Curve III, Dog 3M, 13.9 kilos, received 35 cc. of alcohol. The rates of fall of blood alcohol were as follows: control = 9.7 mg. per cent per hour; after lactate, 1st and 2nd hour = 19; 3rd hour = 16.

increase the rate of alcohol metabolism to the same extent as pyruvate, and the maximum effect that was obtained was sometimes delayed 1 or more hours.

The results are best interpreted in accordance with the oxidation-reduction mechanism. Some effect from the lactate might be expected after high blood levels of both lactate and pyruvate had been established. The increased pyruvate could then be utilized in the dismutation reaction with alcohol, but this reaction would be hindered so long as large amounts of

lactate were continually being absorbed into the blood stream. High lactate levels produced by the dismutation reaction itself would tend to hinder the reaction, and it was previously observed (5) that a second dose of pyruvate was usually less effective than the first.

DISCUSSION

The dismutation reaction observed in these studies occurs when an excess of pyruvate is administered during alcohol metabolism, but there are indications from the data in the literature that this reaction also plays a rôle under normal conditions. The increased blood lactate observed after alcohol ingestion (8) could be derived from the dismutation reaction. The effect of simultaneous carbohydrate metabolism on the rate of alcohol metabolism (5, 9) must be obtained by affecting the rate of oxidation of alcohol to acetaldehyde. Although the dismutation reaction adequately explains many of the relationships between carbohydrate and alcohol metabolism, this reaction alone would not cause the depletion of the carbohydrate reserves observed during alcohol metabolism (10–12), since it does not involve an irreversible utilization of carbohydrate.

The fate of the acetaldehyde produced by the oxidation of alcohol, either directly or through the dismutation reaction, has not been established, but the possibility of acetoin formation was suggested previously (5). The extremely rapid metabolism of acetaldehyde suggests a non-oxidative mechanism or a metabolism that is generally distributed throughout the tissues, rather than an oxidative reaction confined to the liver. The aldehyde mutase (13), which converts 2 moles of acetaldehyde to 1 mole of alcohol and 1 mole of acetic acid, apparently plays no major rôle in the metabolism of acetaldehyde, since the injected acetaldehyde was metabolized without evidence of the formation of any alcohol.

Attempts to demonstrate acetoin formation directly have been unsuccessful. Utilizing a method[1] by which blood acetoin will be detected in a concentration of 0.3 mg. per cent, we have been unable to find acetoin in this amount in dog blood normally or after the administration of pyruvate during or in the absence of alcohol metabolism. These negative results confirm those recently reported by Greenberg (14), but they do not eliminate acetoin as a possible intermediate in alcohol metabolism. A later communication will describe the formation of acetoin from acetaldehyde.

SUMMARY

Evidence is presented indicating that the increased rate of alcohol metabolism resulting from pyruvate administration is due to a coupled oxidation-reduction reaction between the pyruvate and the alcohol.

[1] Stotz, E., unpublished data.

The metabolic relationships normally existing between pyruvate-lactate and alcohol-acetaldehyde were altered by the administration of pyruvate during alcohol metabolism; lactate and acetaldehyde were relatively increased at the expense of pyruvate and alcohol.

The slowest reaction in alcohol metabolism was found to be the oxidation of alcohol itself to acetaldehyde, since a dog was able to metabolize acetaldehyde much more rapidly than it was being formed from alcohol.

Lactate administration usually increased the rate of alcohol metabolism, but the effect was smaller than that observed with pyruvate and was sometimes delayed.

BIBLIOGRAPHY

1. von Euler, H., and Adler, E., *Z. physiol. Chem.*, **226**, 195 (1934).
2. Quibell, T. H., *Z. physiol. Chem.*, **251**, 102 (1938).
3. Green, D. E., and Brosteaux, J., *Biochem. J.*, **30**, 1489 (1936).
4. Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, **32**, 299 (1938).
5. Westerfeld, W. W., Stotz, E., and Berg, R. L., *J. Biol. Chem.*, **144**, 657 (1942).
6. Stotz, E., *J. Biol. Chem.*, **148**, 585 (1943).
7. Briggs, A. P., *J. Biol. Chem.*, **71**, 67 (1926–27).
8. Himwich, H. E., Nahun, L. H., Rakieten, N., Fazekas, J. F., DuBois, D., and Gildea, E. F., *J. Am. Med. Assn.*, **100**, 651 (1933).
9. Clark, B. B., Morrissey, R. W., Fazekas, J. F., and Welch, C. S., *Quart. J. Studies Alc.*, **1**, 663 (1941).
10. Tennent, D. M., *Quart. J. Studies Alc.*, **2**, 263 (1941).
11. Salant, W., *J. Biol. Chem.*, **3**, 403 (1907).
12. Paulesco, N., *Compt. rend. Soc. biol.*, **75**, 588 (1913).
13. Dixon, M., and Lutwak-Mann, C., *Biochem. J.*, **31**, 1347 (1937).
14. Greenberg, L. A., *Quart. J. Studies Alc.*, **3**, 347 (1942).

DETERMINATION OF NICOTINIC ACID

By ROBERT G. MARTINEK,* ERNST R. KIRCH, AND GEORGE L. WEBSTER

(From the Department of Chemistry, College of Pharmacy, University of Illinois, Chicago)

(Received for publication, April 29, 1943)

The discovery of the rôle of nicotinic acid as the antipellagra factor has stimulated work on its determination in various biologicals and food products. The chemical methods of analysis for nicotinic acid (1–10) have been patterned primarily after those which have been used for the determination of pyridine. A number of microbiological methods (11–13) have also been proposed for the determination of nicotinic acid, but they are more time-consuming than the chemical methods. The present investigation was undertaken to evaluate the previously reported chemical and microbiological methods for the determination of nicotinic acid and its amide, and to investigate the possibility of improving the chemical methods of assay.

EXPERIMENTAL

Despite the wide-spread use of aniline in the cyanogen bromide-niacin reaction, this amine has certain very serious disadvantages for use in the assay of niacin. Various amines were allowed to react with cyanogen bromide and niacin in the same manner as for the reaction with aniline. Of the amines investigated, *orthoform* (*m*-amino-*p*-hydroxybenzoic acid methyl ester) was found to produce the most stable color.

Reagents—

1. 1 per cent orthoform (Winthrop) in 95 per cent alcohol. This solution should be kept in the refrigerator in a dark colored bottle.

2. 4 per cent cyanogen bromide in water. A cold, saturated bromine-water solution is carefully decolorized in the cold with 10 per cent sodium cyanide (analytical reagent), measured from a burette. This produces an approximately 4 per cent aqueous solution of cyanogen bromide. Care is taken to prevent having any large excess of the cyanide. This solution, in a dark colored bottle, is kept cold, when not in use, and is stable for long periods.

3. Buffer solution. This is essentially the buffer employed by Waisman and Elvehjem (1), with 95 per cent alcohol substituted for absolute al-

* The experimental data in this paper are taken from a thesis submitted by Robert G. Martinek in partial fulfilment of the requirement for the degree of Master of Science in Chemistry in the Graduate School of the University of Illinois.

cohol. It consists of 988 ml. of water, 15 ml. of 15 per cent sodium hydroxide, 5 ml. of 85 per cent phosphoric acid, and 175 ml. of 95 per cent alcohol.

4. Standard nicotinic acid solution. This solution contains 0.010 gm. of nicotinic acid (Merck) per ml., and should be stored in the refrigerator. For assays, this solution is diluted so that there are 10 γ of nicotinic acid per ml.

Procedure—Solutions equivalent to 5 to 60 γ of nicotinic acid are diluted to 6 ml. with distilled water. To each solution are then added 6 ml. of cyanogen bromide solution and 7 ml. of the buffer solution. All flasks are allowed to stand about 20 minutes (or are heated on the steam bath for 5 minutes with a funnel in the neck of each flask to prevent evaporation), after which time 1 ml. of 1 per cent orthoform solution is added. After

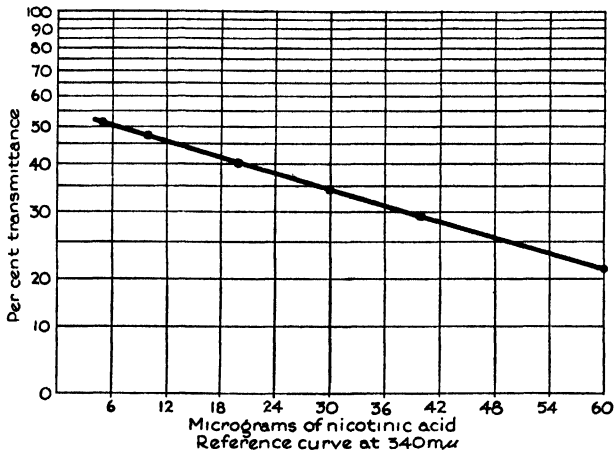


FIG. 1. Standard reference curve for nicotinic acid

5 minutes, and not longer than 15 minutes after the addition of the orthoform, readings are made in a photometer, with the reagents with the exception of the orthoform used as a blank.

In the assay of extracts of various biological materials, however, it may be advisable, in some instances, to follow the suggestion of Melnick and Field (4) of measuring the increment in photometric density obtained by addition of known amounts of nicotinic acid to the extracts.

Standard Curve—A standard reference curve, Fig. 1, is constructed on semilogarithmic paper, with per cent transmission plotted against micrograms of nicotinic acid. Unknown values are interpolated from the standard curve.

Influence of pH on Reaction—Pure aqueous solutions of nicotinic acid

were tested and the results are shown in Table I. It can be seen that, for 100 per cent recovery, the pH must be maintained within the limits of 6.2 to 7.05. If the solution becomes acid to a pH of 3.75, as much as 95 per cent loss occurs, whereas, if the pH becomes as high as 8, about a 35 per cent loss occurs.

TABLE I

Effect of pH on Reaction

Each sample contained 60 γ of nicotinic acid.

Sample No.	pH after reaction	Niacin found	Per cent recovered	Sample No.	pH after reaction	Niacin found	Per cent recovered
		γ				γ	
1	3.75	3.0	5.0	8	6 69	60.0	100.0
2	4.35	16.0	26.7	9	6.83	60.0	100.0
3	4.89	21.5	35.8	10	7.05	60.0	100.0
4	5.20	23.5	39.2	11	7.11	50.5	84.2
5	5.41	32.0	53.5	12	7.25	44.5	74.2
6	5.82	53.5	89.2	13	7.55	42 5	70.8
7	6.20	60.0	100.0	14	7.95	39.0	65.0

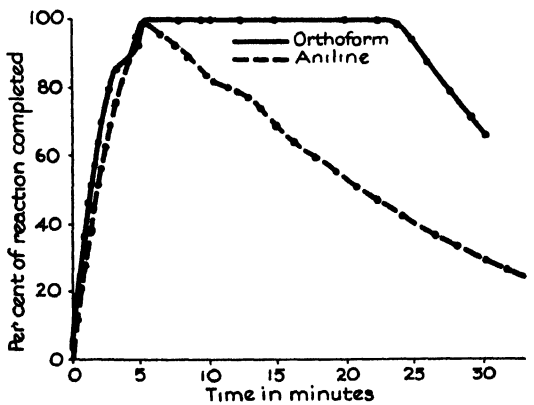


FIG. 2. Comparative stability of color found with aniline and orthoform

Time Required for Development of Maximum Color—Two series of experiments with aniline and orthoform respectively were carried out on 60 γ of nicotinic acid. Measurements of the per cent transmittance were made at intervals of from ½ to 30 minutes after the addition of the amine. The results are represented in Fig. 2. It can be seen that the color produced with aniline reaches a maximum in 5 minutes and then rapidly fades. The orthoform-produced color, however, reaches a maximum in 5 minutes and is stable for at least 15 minutes.

Specificity of Reaction—The one known vitamin, other than nicotinic acid, that contains the pyridine ring is pyridoxine or vitamin B_6. This vitamin was found to yield no appreciable color in the cyanogen bromide color reaction with orthoform as the reagent. As many as 60 γ of 2-aminopyridine produced only a very slight color in contrast to the color produced when aniline is used as the reagent, as reported by Waisman and Elvehjem (1).

Assay of Nicotinic Acid in Yeast by Means of 1 Per Cent Orthoform Method—Yeast extracts, prepared according to the technique of Waisman and Elvehjem (1) and decolorized with Darco G-60, were assayed for

TABLE II

Apparent Nicotinic Acid Values in Yeast

Sample No.	4 per cent aniline	1 per cent orthoform	Microbiological assay
	mg. per 100 gm.	*mg. per 100 gm.*	*mg. per 100 gm.*
1	2.90	2.97	2.12
2	2.43	3.18	3.31
3	2.30	2.10	2.19
4	3.54	3.11	3.41

TABLE III

Determinations of Nicotinamide in Commercial Sample (25.0 Mg. per Liter on Label)

Lot sample No.	Found	
	4 per cent aniline	1 per cent orthoform
	mg. per l.	*mg. per l.*
1	23.0	23.0
2	23.5	21.5
3	22.0	21.0
4	21.0	23.0

nicotinic acid with orthoform as the chromogenic amine. For the purpose of evaluation and comparison, the chemical assay with aniline, as modified by Waisman and Elvehjem (1), and the Landy and Dicken (13) microbiological assay were performed on the same extracts. Results are shown in Table II.

Assay of Nicotinic Acid in Commercial Sample by Means of 1 Per Cent Orthoform Method—An intravenous solution, containing, according to the label, dextrose U.S.P. 50.0 gm., sodium chloride U.S.P. 9.0 gm., thiamine hydrochloride U.S.P. 3 mg., riboflavin 3 mg., nicotinamide 25 mg. per liter, was assayed for nicotinic acid.

The assay procedure was as follows: The color due to the riboflavin was first destroyed by exposure of a portion of the solution to the light of a 100 watt bulb for 48 hours. A portion of the solution was then subjected to hydrolysis with 0.1 N HCl in an autoclave at 15 pounds for 15 minutes in order to convert all the amide to the free acid. The resulting hydrolysate was made up to volume in a volumetric flask, and then aliquots equivalent to from 25 to 50 γ of nicotinic acid were assayed with orthoform as the amine.

The same procedure of assay was then repeated with 1 ml. of 4 per cent aniline in alcohol in place of the 1 ml. of 1 per cent orthoform in alcohol. The results are shown in Table III.

SUMMARY

The use of orthoform as a chromogenic amine in the cyanogen bromide-niacin reaction has been studied and found to make the method less critical with respect to pH control and time of comparison of the color, with no loss of accuracy.

BIBLIOGRAPHY

1. Waisman, H. A., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 221 (1941).
2. Bandier, E., and Hald, J., *Biochem. J.*, **33**, 264 (1939).
3. Shaw, F. E., and McDonald, O. A., *Quart. J. Pharm. and Pharmacol.*, **11**, 380 (1938).
4. Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, **134**, 1 (1940); **135**, 53 (1940).
5. Pearson, P. B., *J. Biol. Chem.*, **129**, 491 (1939).
6. Swaminathan, M., *Indian J. Med. Research*, **26**, 427 (1938).
7. von Euler, H., Schlenk, F., Heiwinkel, H., and Hogberg, B., *Z. physiol. Chem.*, **256**, 298 (1938).
8. Harris, L. J., and Raymond, W. D., *Chem. and Ind.*, **58**, 652 (1939).
9. Kodicek, E., *Biochem. J.*, **34**, 712, 722 (1940).
10. Arnold, A., Schreffler, C. B., and Lipsius, S. T., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 62 (1941).
11. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
12. Isbell, H., *J. Biol. Chem.*, **144**, 567 (1942).
13. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **24**, 1086 (1942).

THE RELATIONSHIP OF EXTINCTION TO WAVE-LENGTH IN TURBID SERA AND OTHER SUSPENSIONS

By OLIVER HENRY GAEBLER

(From the Department of Laboratories, Henry Ford Hospital, Detroit)

(Received for publication, May 3, 1943)

The purpose of this study was to determine whether the turbidity of blood sera, measured in a spectrophotometer, is systematically related to wave-length, so that the error introduced at a point where extinction[1] of an added dye or a colored constituent of the serum is measured might be computed from determinations of turbidity made in other regions of the spectrum. The writer was interested in applying such information in determinations of plasma volume, but the results obtained are also of interest in other connections.

Suspensions of coarse particles, which reflect light, may have the same extinction at widely different wave-lengths. Minute particles, on the other hand, produce excessive scattering of the blue end of the spectrum. This is mathematically expressed in Rayleigh's law (1). For class purposes one may demonstrate the fact without a spectrophotometer by nearly filling a 100 cc. flask with water and adding 1 cc. each of the silver nitrate and ammonium thiocyanate solutions used for determination of chloride in urine. The fine precipitate which forms in the absence of nitric acid looks bluish in reflected light. But if the flask is held between the observer and the white bowl of a light, immediately after the thiocyanate is added, the image of the bowl will change through yellow to red before it is obscured entirely.

In sera, turbidities produced by physiological lipemia and other causes are due to particles which are small enough to scatter light in this way. That a linear relationship might exist between the logarithm of extinction and the negative logarithm of wave-length was suspected, since there are formulae in which the fraction of light scattered by minute particles and the turbidity are related to the inverse fourth power of the wave-length (1). The expected relationship was observed in the instances discussed below. Less convenient though equally systematic relationships were observed in the cases of other suspensions or more concentrated preparations.

[1] The term optical density is commonly used in place of extinction to signify $\log (I_0/I)$, I_0 being the intensity of incident light and I the intensity of transmitted light.

EXPERIMENTAL

Suspensions of protein precipitated with sulfosalicylic acid and sodium sulfate (Exton's reagent) may be considered first. This artificial suspension permits direct measurements of turbidity in parts of the spectrum where hemoglobin and bilirubin interfere with measurements in sera. The procedure was as follows: Three dog sera which contained very little hemoglobin were diluted 120 to 200 times with 0.8 per cent sodium chloride solution. 1 volume of diluted serum was mixed with 1 volume of Exton's reagent, which contained 50 gm. of sulfosalicylic acid and 88 gm. of anhydrous sodium sulfate per liter, but no indicator. The extinction at various wave-lengths was measured in a Beckman spectrophotometer (2). The blank consisted of equal parts of 0.8 per cent sodium chloride solution and Exton's reagent. It was necessary to mix the suspensions before each measurement, for they flocculate and settle appreciably. The results are shown in Fig. 1, and indicate a linear relationship between the logarithm of extinction and the negative logarithm of the wave-length, from 1000 mμ in the infra-red to 350 mμ in the ultraviolet. Below the latter point sulfosalicylic acid solutions of this concentration do not transmit, and for some distance above it the concentration of this substance in the blank and the unknown must be the same.

Serum turbidities which are not due to blood cells or other large particles that a centrifuge will remove also follow the above rule. If hemolysis is not sufficient to interfere, the relationship between the negative logarithm of wave-length and the logarithm of density is linear between 1000 and 600 mμ. This is shown in Fig. 2. At shorter wave-lengths the spectra of bile pigments and oxyhemoglobin (which is readily found in "unhemolyzed" sera) interfere with direct measurement of turbidity. Evans' blue shows maximal absorption at 620 mμ (3), and dog sera containing it show maximal absorption at about 630 mμ. Thus Fig. 2 covers the region of interest in this case.

In the concentrations commonly employed, Evans' blue in serum shows negligible absorption at 750 mμ, especially if the sera are diluted, as in a method which the writer uses. With salt solution as a blank, one can therefore measure the density of dyed sera at 1000, 900, 800, and 750 mμ, plot the values as in Fig. 2, and graphically determine the correction to be subtracted from the observed density at 630 mμ by projecting the line to that point on the graph. The writer found this method applicable to many turbid sera, but two facts limit its general usefulness. Many instruments are not adapted for measurements in the infra-red, and compounds such as oxyhemoglobin have irregular absorption spectra in this region. The graphical method must then give way to an algebraic one in which factors

derived from the data in Fig. 2 are employed. Details of this will be published elsewhere, and only the principle need be mentioned here.

Two colored substances with overlapping absorption spectra can be determined in the presence of one another under certain conditions. The substances must not react with each other, and the ratio between extinctions at two points in the spectrum must be quite different for each substance. In Fig. 2 it is apparent that the slope of the lines is very similar. In other words, the ratio of extinctions at two or more points in the spectrum may be as constant for a given turbidity as for a given color. Under these circumstances determination of a colored substance in the presence of unavoidable turbidity can be made in the same manner that one determines two colored substances in the presence of one another (3).

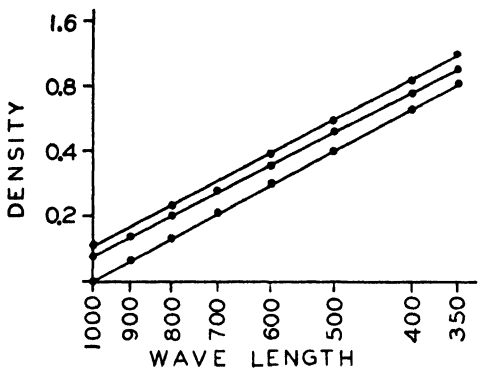


FIG. 1

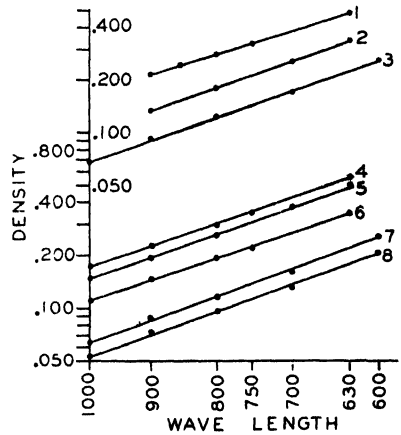


FIG. 2

FIG. 1. Suspensions prepared by precipitating diluted sera with sulfosalicylic acid and sodium sulfate were compared with a blank containing the same concentration of the precipitants. A linear relationship between the logarithm of optical density and the negative logarithm of wave-length was observed.

FIG. 2. The relationship between the logarithm of optical density and the negative logarithm of wave-length is shown in the case of turbid sera. Curves 1, 2, and 3 are plotted on the upper scale. The material included four turbid sera from dogs in the postabsorptive state, which had received 42 per cent of their calories in the form of fat (Curves 2, 3, 4, and 7), and two dog sera which became turbid on standing 12 hours in the refrigerator (Curves 1 and 6). In all of these cases 1 volume of serum was diluted with 1 volume of 0.8 per cent sodium chloride solution, and the salt solution was used as the blank in the spectrophotometric readings. In one case (Curve 5) turbid dog serum obtained 3 hours after a high fat meal was diluted as already stated and read against similarly diluted clear serum obtained before the meal. Curve 8 represents an undiluted, turbid dog serum read against the same serum diluted with an equal volume of salt solution.

SUMMARY

Dog sera in which turbidity was due to physiological lipemia and other causes were examined in a photoelectric spectrophotometer. Analysis of the data showed that the logarithm of the extinction was related in a linear manner to the negative logarithm of the wave-length. This was also true in the case of turbid suspensions made by precipitating greatly diluted serum with sulfosalicylic acid and sodium sulfate. The value of this relationship in correcting for turbidity is discussed.

BIBLIOGRAPHY

1. Wells, P. V., *Chem. Rev.*, **3**, 331 (1926–27); see Equation 22.
2. Cary, H. H., and Beckman, A. O., *J. Optical Soc. Am.*, **31**, 682 (1941).
3. Gibson, J. G., 2nd, and Evans, W. A., Jr., *J. Clin. Inv.*, **16**, 301 (1937).

THE COLORIMETRIC ESTIMATION OF AMINO NITROGEN IN BLOOD*

By ELIZABETH G. FRAME, JANE A. RUSSELL, AND ALFRED E. WILHELMI

(From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven)

(Received for publication, May 4, 1943)

The best available methods for the determination of amino acid nitrogen, the nitrous acid manometric and the ninhydrin manometric and titrimetric procedures, require even in their submicro modifications at least 40 γ of amino nitrogen per sample and are more satisfactory when larger amounts are used. They are therefore unsuited for use with very small samples of blood, such as may be obtained serially from small laboratory animals. To fulfil this requirement we have developed a colorimetric method which may be used to determine 4 to 40 γ of amino nitrogen per sample, with a precision usually of 1 to 2 per cent. Duplicate analyses may be made upon 0.2 ml. of blood. While the method is not as specific as the ninhydrin reaction, its simplicity and convenience make it useful in circumstances when the greatest precision is not required.

The method depends upon the combination of amino nitrogen groups with β-naphthoquinone sulfonate in alkaline solution to form highly colored compounds, orange-red in acid solution, which may be determined colorimetrically (1). The method originally described by Folin for the estimation of amino acid nitrogen in blood required 2 ml. of blood for duplicate determinations and 20 to 24 hours for color development. According to Van Slyke and Kirk (2) and others, the estimation of amino nitrogen by this procedure was incomplete. The accuracy of the method was much improved by Danielson (3), who modified the conditions of color development and the reagents used for bleaching the excess naphthoquinone. Sahyun (4) hastened the reaction and probably made it more complete by heating the reaction mixture during color development. Although he found it possible to apply the method to 0.2 ml. of blood (for duplicates), he recommended using larger samples. In all of these procedures a Duboscq type of colorimeter was employed, with resulting inaccuracies due to a large blank color and to differences in hue produced by different amino acids and amino acid mixtures. The specificity of the reaction as carried out by the modified procedures was not investigated.

* Part of the material in this paper was included in a thesis presented by Elizabeth G. Frame to the faculty of the Graduate School, Yale University, in partial satisfaction of the requirements for the degree of Doctor of Philosophy.

The present procedure embodies with modifications certain of the conditions recommended by Danielson and by Sahyun. The absorption spectra of the amino acid-napththoquinone compounds have been studied, and the method has been adapted to the photoelectric colorimeter. The individual amino acids were compared quantitatively, and most of them were found to give nearly identical results with respect to their free amino nitrogen content. In addition a number of other nitrogen-containing substances have been tested in order to determine the specificity of the reaction under the conditions described here. The method applied to blood has shown good agreement with the Van Slyke nitrous acid method and satisfactory recovery of added amino acid nitrogen.

EXPERIMENTAL

Reagents and Apparatus—

Sodium β-naphthoquinone-4-sulfonate, 0.5 per cent solution, freshly prepared within an hour before use.

Sodium tetraborate (borax), 1 per cent solution.

Sodium hydroxide, 0.1 N.

Phenolphthalein, 0.25 per cent alcoholic solution.

Acid-formaldehyde; 3 parts of 1.5 N hydrochloric acid, 1 part of glacial acetic acid, and 4 parts of 0.15 M formaldehyde (11.3 ml. of 40 per cent formaldehyde diluted to 1 liter).

Sodium thiosulfate, 0.1 N.

Stock standard amino acid solution; 0.2 mg. of amino nitrogen per ml., dissolved in a solution of 0.2 per cent sodium benzoate in 0.7 N hydrochloric acid.

In the method as described an Evelyn photoelectric colorimeter is employed in conjunction with Rubicon Filter 490 or, in certain circumstances, Filter 520. The entire procedure may be carried out in test-tubes calibrated at 15 ml., or in calibrated colorimeter tubes.

Color Development—To a sample of the unknown solution, containing preferably 8 to 30 γ of amino nitrogen, add water if necessary to a volume of about 5 ml. Add 1 drop of phenolphthalein solution to the sample and then add 0.1 N sodium hydroxide drop by drop until a permanent pink color is established. Adjust the pH by adding 1 ml. of the borax solution (or of a borate buffer, pH 9.2 to 9.4). Then add 1 ml. of the freshly prepared naphthoquinone solution. Mix the contents of the tube, place it immediately in a briskly boiling water bath, and heat for 10 minutes. After heating, place the tube in cold water for about 5 minutes. To acidify the mixture and to decolorize the excess naphthoquinone first add 1 ml. of the acid-formaldehyde solution and mix, and then add 1 ml. of 0.1 N sodium thiosulfate. Make up to 15 ml. with water, mix, and let stand

for 10 to 30 minutes. Read in a colorimeter, using preferably a 480 or 490 mμ filter. For the initial setting of the colorimeter a reagent blank should be prepared. Under the conditions described here, the bleaching of the excess quinone is not quite complete, the blank being pale yellow in color. However, if the conditions of analysis are well standardized, the absorption of the blank is quite constant in value. Small variations may occur owing to differences in heating and in the time and temperature of cooling; so that for accurate results blank tubes should be prepared simultaneously with each group of unknown samples. Duplicate determinations by this procedure agree almost invariably within 3 per cent and usually within 1 or 2 per cent.

The reaction must be carried out at pH 9.2 to 9.4, much less color being developed outside this range. A drop or two of 0.1 N sodium hydroxide added in excess during neutralization does not affect color development, but incomplete neutralization (to a faint or transient pink color with phenolphthalein) leads to low results. The volume of the sample should not exceed 5 ml. by more than a few tenths of a ml.; so that if the solution to be analyzed is strongly acid, more concentrated alkali should be used before the final neutralization with 0.1 N sodium hydroxide.

Heating 10 minutes was found sufficient for full color development with mixtures of amino acids as found in blood filtrates and casein hydrolysates and with most of the pure amino acids. About 90 per cent of the color is obtained after 5 minutes, and no additional color is developed after 15 minutes. The blank color is, however, considerably increased with longer heating. Accordingly, the 10 minute period has been used in routine determinations. Heating 10 minutes is not quite sufficient for full color development with two amino acids, arginine and leucine; a 15 minute period might therefore be recommended if an unusually high content of either of these amino acids was present in the sample being analyzed.

The naphthoquinone solution must be freshly prepared, at least within an hour of being used. On standing, in contact with alkali especially, changes take place which make incomplete the subsequent decolorizing of the blank. For this reason the color reagent should be added to the samples not more than a few minutes before the heating is begun, and the length of the cooling period should be short. The bleaching of the excess quinone is complete in about 10 minutes. The amino acid-quinone complex is only slowly affected, beginning to fade perceptibly about 30 minutes after the addition of the thiosulfate.

After the addition of acid and thiosulfate the colored solutions usually remain perfectly clear for some hours. Occasionally some cloudiness may develop soon after bleaching, owing probably to the precipitation of sulfur from the thiosulfate in acid solution. It has been found by others using

the method that the development of cloudiness may be prevented by the addition of a drop of 4 per cent Duponol (WA) to the mixture before acidification.

The presence of traces of chromic acid on the glassware leads to irregular results. If a chromic acid cleaning agent has been used, the glassware should be well rinsed in an alkaline wash solution.

Color Measurement—Since the accuracy of photometric methods depends upon a correct choice of light filter, it is necessary to know the spectral characteristics of the colored substance to be measured. Moreover, in the estimation of amino nitrogen one is usually dealing with mixtures of different substances, and assurance is required that the several substances yield compounds with similar absorption maxima. Accordingly, the absorption spectra of the colored substances produced in combination with β-naphthoquinone sulfonate by seventeen amino acids and by blood filtrates were determined with a Coleman universal spectrophotometer. Readings were made at 10 mμ intervals over the range 360 to 700 mμ. The amino acids tested were alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, leucine, lysine, methionine, ornithine, phenylalanine, serine, threonine, tryptophane, and tyrosine. The samples contained about 16 γ of amino nitrogen and were dissolved in acid benzoate solution as described in the preparation of the standard curve.

The absorption spectra of the amino acid compounds all resemble one another closely. A few of the curves representative of most of the amino acids studied are presented in Figs. 1 and 2, the less typical curves in Fig. 3. The absorption maximum very near 480 mμ is characteristic of all the curves, which differ mainly in the relative amounts of absorption shown in the descending limbs. None of these differences is great enough to permit the estimation of any single amino acid in a mixture; they are responsible, however, for the variations in shade of the different colors as observed by the eye. The diamino acids lysine and ornithine exhibit almost identical curves, having a notched peak with one maximum at 480 mμ and the other at 500 mμ. Hydroxyproline is also exceptional for its low degree of absorption and for a second, lower peak at 450 mμ. The curve for proline was not determined, but it is probably similar to that of hydroxyproline, since the analytical behavior of the two compounds is almost identical. The absorption spectrum of the color developed with blood filtrates conforms closely to that of the major group of the amino acids.

From the absorption spectra it is clear that the filter of choice for the measurement of this color would be one with maximum light transmission at 480 mμ. We have successfully used Rubicon Filter 490, which transmits light of wave-lengths 465 to 530 mμ, and under certain circumstances, Filter 520 (495 to 550 mμ). The latter filter is not particularly recom-

mended; but data on its use are included because at present a full selection of filters may not be available, and because in some early work we employed this filter. As would be expected from the absorption spectra, Filter 520 is less sensitive to differences in amino nitrogen content and more sensitive to the differences in relative absorption shown by the individual amino acids. When constant amounts of the single amino acids are compared with an equivalent standard containing glycine and glutamic acid, the variations in amount of color with Filter 520 are mostly between 85 and 115 per cent of the standard and are about equally divided in positive and

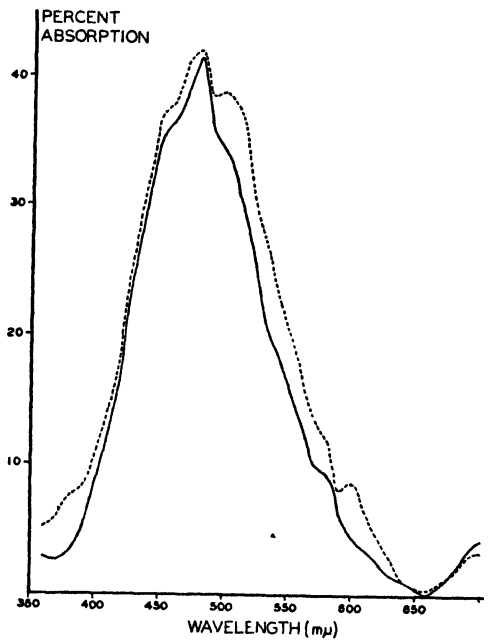


FIG. 1. The absorption spectra of the compounds formed by the reaction of glutamic acid (solid line) and of alanine (broken line) with β-naphthoquinone sulfonate, corrected for the absorption of the reagent blank.

negative directions (Table I). The color developed by complete mixtures of amino acids might therefore be expected to be fairly comparable, when measured with Filter 520, to that of a mixed standard of the same amino nitrogen content. In a series of fifteen determinations of amino nitrogen in blood filtrates which were made with Filters 490 and 520, the results obtained at the two wave-lengths disagreed only slightly. The differences ranged from −2.3 to +5.9 per cent of the values obtained at 490 mμ. The average difference was +1.9 per cent, or, in terms of amino nitrogen at the usual levels in blood, about 0.2 mg. per cent. Filter 520 might therefore

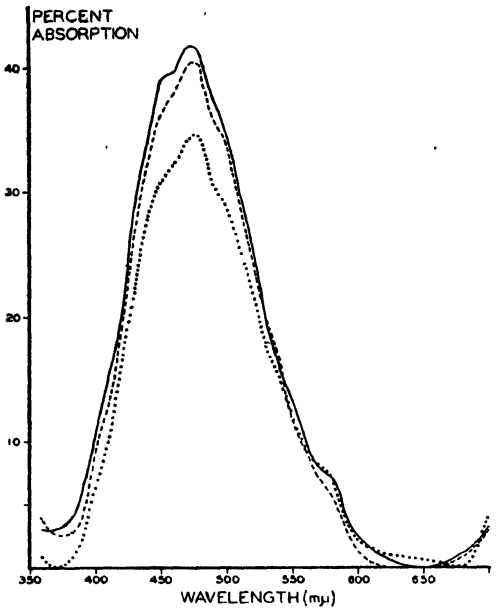


FIG. 2. The absorption spectra of the compounds formed by the reactions of methionine (solid line), tyrosine (broken line), and histidine (dotted line) with β-naphthoquinone sulfonate.

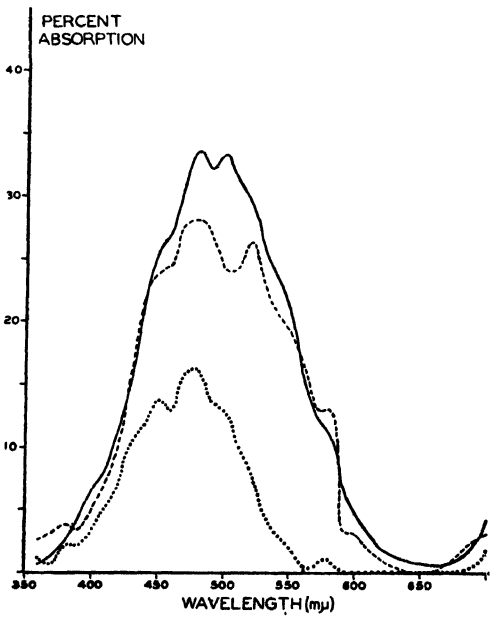


FIG. 3. The absorption spectra of the less typical amino acid compounds of β-naphthoquinone sulfonate with lysine (solid line), threonine (broken line), and hydroxyproline (dotted line).

be used for comparative measurements of similar blood filtrates with but little loss in accuracy.

Standard Curve—Any one of a number of amino acids can be used as a standard, if the colors of standard and unknown are compared at 490 mμ (Table I). Danielson recommended as a standard an equimolar mixture of glycine and glutamic acid, because its color closely matches in shade that formed by blood filtrates. This mixed standard has been used in most of our work, primarily because in the early phases of the investigation we did not have complete spectroscopic data on the colors produced in the reaction, and we wished to have as close correspondence as possible between the colors of the standard and unknown solutions. The stock solution of the mixed standard consists of 53.6 mg. of glycine and 105 mg. of glutamic acid made to 100 ml. in a solution of 0.2 per cent sodium benzoate in 0.07 N hydrochloric acid; it contains 0.200 mg. of amino nitrogen per ml. For use as a standard to be prepared simultaneously with unknowns or in the preparation of a standard curve, the stock solution may be diluted as required either with water or with acid benzoate. If water is used, the reagent blank need contain only water, since small amounts of acid benzoate do not affect the light absorption of the blank.

The color produced in this reaction is sufficiently reproducible, for most purposes, for preparing a standard curve and the results of analyses may be calculated by reference to this curve. For this calculation, the galvanometer readings (G) obtained from the Evelyn colorimeter are first converted to L (density) values by reference to the L/G table. The amount of amino nitrogen present in the sample may then be calculated from this value by the use of a regression equation derived from the standard data. Fig. 4 shows the regression lines obtained from glycine-glutamic acid standards (4 to 30 γ) read with Filters 490 and 520. Micrograms of amino nitrogen are plotted against L ($= 2 - \log G$). There is an exact straight line relationship between the amount of amino nitrogen and intensity of color developed throughout the accurate range of the colorimeter.

Comparison of Individual Amino Acids—Twenty-one amino acids and some derivatives of amino acids were compared with the glycine-glutamic acid standard in the reaction carried out as described above. Readings were made with Filters 490 and 520. Three or more determinations were made in each instance, usually simultaneously with standards. The results are presented in Table I. Most of the individual amino acids gave nearly identical results with respect to their α-amino nitrogen content when Filter 490 was used. Exceptions to this agreement were the following: Both amino groups of lysine and ornithine appeared to react quantitatively with the naphthoquinone reagent. Arginine is somewhat slow to react, giving 90 per cent of its color after a 10 minute heating period but yielding

theoretical results for its α-amino nitrogen content after 15 minutes of heating. Histidine, leucine, and valine gave 90 to 93 per cent of their theoretical α-amino nitrogen content; with leucine only, the color was increased (to 96 per cent) by further heating. The color produced with the latter amino acids was more yellow than in the majority of cases, and their absorption curves exhibit a sharp descent on the red side from the peak at 480 mμ. It is probable that these amino acids would yield more nearly theoretical values if a filter with its principal transmission at 480 mμ instead of at 490 mμ were used. The nitrogen of proline and hydroxyproline, which is in the imino form, gave only half the color to be expected from amino nitrogen. Interestingly, with these two compounds the reac-

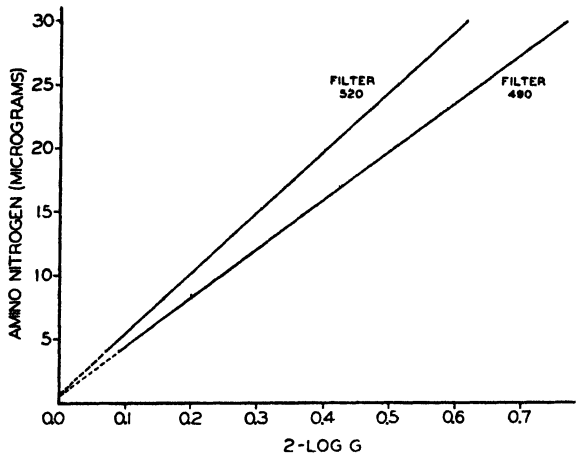


FIG. 4. The relationship of color intensity ($L = 2 - \log G$) to the amount of amino acid nitrogen in an equimolar mixture of glycine and glutamic acid. The regression equations of the standard curves, where Y = micrograms of amino nitrogen, are as follows: with Filter 490 $Y = 38.34 + 0.594$; with Filter 520 $Y = 47.53 + 0.671$.

tion is very rapid, being completed in the cold in the course of minutes. Thyroxine yields a colored precipitate on acidification, but at low concentrations the precipitation could be largely delayed in the presence of Duponol, so that an approximate reading of the color, about 85 per cent of theoretical, could be obtained. Alanine gave somewhat more color than the standard mixture.

Peptide and amide nitrogen do not appear to react with the naphthoquinone reagent. Moreover, the free α-amino nitrogen of the amides and peptides tested, except glutathione, did not develop full color. The reason for this apparent inhibition is not clear. Among the peptides, the proximity of a free carboxyl group may influence the intensity of color. Tyrosylglycinamide, which has no free carboxyl group, was the least

chromogenic, while glutathione, an abnormal peptide in that it contains free amino and carboxyl groups on the same carbon atom, was fully reactive.

Reactions of Other Nitrogenous Substances—A number of nitrogen-containing substances other than amino acids were tested for the possibility

TABLE I

Determination of Individual Amino Acids

12 γ of amino nitrogen were used in each sample.

Amino acid	Amino nitrogen measured, per cent of standard		Amino acid	Amino nitrogen measured, per cent of standard	
	With Filter 490	With Filter 520		With Filter 490	With Filter 520
Glycine-glutamic acid standard	100	100	Threonine	100	109
			Thyroxine	86	75 (Ppts.)
Alanine	108	108	Tryptophane	101	101
Arginine	92	86	Tyrosine	100	89
" heated 15 min.	100	97	Valine	93	70
Aspartic acid	100	116	Amino acid derivatives*		
Cysteine	100	104			
Cystine	99	107	Asparagine	86	78
Glutamic acid	100	96	Glutamine	70	68
Glycine	100	108	Glutathione	100	94
Histidine	92	88	Glycylglycine	81	90
Hydroxyproline	51	36	Leucylglycylglycine	66	55
Leucine	90	77	Tyrosylglycinamide	50	54
" heated 15 min.	96	85	Glycine anhydride	0	0
Lysine	216	236	Hippuric acid	0	0
Methionine	100	88	Carbobenzoxytyrosinamide	0	0
Ornithine	200	234			
Phenylalanine	100	85	Carbobenzoxyglutamyltyrosine	0	0
Proline	45	37			
Serine	99	94			

* To calculate the results of these analyses, the compounds were considered to contain one free amino group per molecule. In the last four cases, larger amounts than 12 γ of nitrogen were also used; less than 1 per cent of the nitrogen in each compound reacted as amino nitrogen.

of their interference with the determination of amino acids, or because of their theoretical interest. The results of most of these tests are shown in Table II. Under the conditions of this test, the quinone appeared to react not at all or to a very slight extent only with amide nitrogen, peptide nitrogen, tertiary amines, the unsaturated pyrimidine, pyridine, purine, imidazole, and thiazole rings, guanidino groups, urea, creatine, creatinine.

TABLE II

Reactions of Substances Other Than Amino Acids (Filter 490)

Substance	Amount of sample	Nitrogen* in sample	Amino nitrogen determined	
	mg.	*γ*	*γ*	*per cent of N* present*
Ammonia	0.195	16	7	58 (Variable)
Urea	0.535	250	1.3	0.5
Creatine	2.34	750	1.9	0.2
Creatinine	2.02	750	1.7	0.2
Uric acid	0.150	50	14.1	28
Allantoin	0.710	250	33	13
Uracil	0.400	100	0.0	0 0
Adenine sulfate	0.986	375 (75)	0.0	0.0
Guanine chloride	0.401	150 (30)	1.7	1.1 (6)
Hypoxanthine	0.121	50	0 0	0.0
Thiamine chloride	5.37	1000 (250)	12.0	1.2 (5)
Nicotinic acid	2.20	250	0.0	0.0
Primary aliphatic amines				
Hydroxylamine chloride	0.080	16	4	25 (Variable)
Tyramine	0.118	12	7 6	63
Histamine hydrochloride	0.126	36 (12)	8 9	(74)
Benzedrine sulfate	0.270	20	12 9	65
Cobefrin chloride	0.314	20	18 0	90
Secondary amines				
Dimethylamine chloride	1.84	316	23	7 (Variable)
Ephedrine sulfate	0.290	20	9 1	46
Adrenalin chloride	0 260	20	20	100 (Approximate)
Tertiary amines				
Trimethylamine chloride (see also Procaine)	1.70	250	4	2 (Variable; possibly contaminated with ammonia)
Amides and substituted amides (—CO—NH—)				
Acetamide	0.140	33	0.0	0.0
Acetanilide	0.193	20	0.0	0.0
Acetylsulfanilamide	0.266	(20)	2.1	(10)
Succinylsulfathiazole	0.508	(20)	1.1	(5)
Primary aromatic amines				
Aniline	0.105	16	16.0	100
p-Aminobenzoic acid	0.117	12	11.2	93 (Ppts.)
p-Aminoacetophenone	0.194	20		(Insoluble)
Sulfanilic acid	0.300	20	25.1	125
Procaine chloride (also contains a tertiary amine group)	0 232	24 (12)	10.1	(84)

TABLE II—*Concluded*

Substance	Amount of sample	Nitrogen* in sample	Amino nitrogen determined	
	mg.	γ	γ	*per cent of N* present*
Orthoform..	0.233	20	9.5	47
Arsphenamine dichloride	0.316	20	35.7	178
Mapharsen..	0.317	22 5	35 3	157
Sulfanilamide. ..	0.246	(20)	19 2	(96)
Sulfapyridine	0.356	(20)	23.0	(115)
Sulfathiazole	0.365	(20)	24.6	(123)
Sulfadiazine	0.357	(20)	22.3	(112)
Sulfamerizine	0 378	(20)	23.7	(118)
Sulfaguanidine	0.360	(20)	17.8	(89)

* Figures in parentheses refer to amino nitrogen and per cent of amino nitrogen. For the sulfonamide derivatives, these figures refer to the *p*-amino group on the benzene ring.

and the amino purines. Besides reacting with all amino acids, the quinone yields similar but less intense colors with ammonia and primary aliphatic amines and some secondary amines. Primary aromatic amines react easily, yielding as much color as the amino acids; often the reaction takes place quickly at room temperature. In addition, uric acid and allantoin form compounds which are red in acid solution. When these substances are compared with amino acid standards at 490 mμ, 28 per cent of the uric acid nitrogen and 13 per cent of the allantoin nitrogen appear as amino nitrogen. At 520 mμ, the equivalence is 38 and 69 per cent respectively.

The principal substances which occur naturally and which might lead to erroneous results in the estimation of amino acid nitrogen by this method are ammonia, uric acid, and allantoin. In blood and most tissues, none of these compounds is ordinarily in sufficient quantity to interfere seriously. Uric acid, the most concentrated of these compounds in blood, yields a color equivalent to 0.1 mg. per cent of amino nitrogen when it is present in a concentration of 1.0 mg. per cent. A correction may be applied when amino acid nitrogen is determined in bloods very high in uric acid. This method of estimating amino nitrogen could be applied to human urine only after the removal of ammonia[1] and of uric acid. In the urine of animals, allantoin would also interfere.

Since aromatic amines react with the quinone reagent, the presence in blood of unconjugated aminobenzenesulfonamide derivatives would aug-

[1] Permutit, which has been recommended for the removal of ammonia prior to the estimation of amino acids, was found in one experiment to remove a large part of the amino nitrogen from a mixture of amino acids.

ment the amino acid values obtained by this method. However, only 5 to 8 per cent of the aminosulfonamides by weight is recorded as amino nitrogen (a level of 10 mg. per cent of free sulfanilamide in blood being equivalent to 0.8 mg. per cent of amino acid nitrogen); so that interference from this source is not extremely serious. Appropriate correctoins may of course be applied when necessary. The conjugated sulfonamides, such as acetylsulfanilamide or succinylsulfathiazole (sulfasuxidine), are relatively unreactive. The arsenicals, of which arsphenamine and mapharsen were tested, are not found in blood to an appreciable extent (5).

Application to Blood

Protein Precipitants—A number of blood protein precipitants have been tested as to which ones yield filtrates suitable for the application of this method. Tungstic acid filtrates have been found so far to be the most satisfactory. Zinc hydroxide filtrates gave amino nitrogen values which were about half those obtained with tungstic acid filtrates and recoveries of added amino acids were low, indicating that some of the amino acids were carried down with the protein. Trichloroacetic, metaphosphoric, and sulfosalicylic acids develop a considerable amount of color with the naphthoquinone reagent, with the result that the blank readings are too high for use with the photoelectric colorimeter. Since the presence of copper ions also increases the blank color and since many amino acids have insoluble copper salts, copper hydroxide and copper tungstate are also unsuitable precipitants.

The tungstate filtrates may be prepared in the usual way, or, when only small amounts of blood are available, they are conveniently made by the procedure described by Shipley and Long (6). In this method, 0.2 ml. of blood is diluted with 10.0 ml. of N/70 sulfuric acid; the proteins are then precipitated by the addition of 0.2 ml. of 10 per cent sodium tungstate and removed by centrifugation. 4 ml. of such a filtrate of normal blood are a suitable amount to use, allowing duplicate determinations to be made on 0.2 ml. of blood. The reagent blank need contain no tungstate. Since the presence of more than traces of tungstate interferes with color development, filtrates containing a large amount of tungstate which has escaped precipitation may not be used, but normally no interference is to be expected from this source. The amino acid content of tungstate filtrates kept under refrigeration remains constant for several days.

Inasmuch as the amino acid content of the red blood cells may be much greater than that of the plasma, it may be preferable at times to carry out determinations on plasma itself. The same procedure is followed in this case as in the determination of amino acids in whole blood.

Recovery of Added Amino Acids—A mixture of amino acids in the form

of casein hydrolysate was added to a series of 0.2 ml. samples of rat blood before protein precipitation with tungstic acid. The amino acid content

TABLE III

Recovery of Amino Acids (Casein Hydrolysate) Added to Blood

Amino N in blood	Amino N added to 0.2 ml blood	Amino N in blood containing added N	Recovery of added amino N
mg. per cent	γ	*mg per cent*	*per cent*
16.1*	20.2	25.4	92
16.1*	20.2	25.2	90
14.2*	18.0	23.8	107
14.2*	18.0	23.5	103
12.4	45.6	35.4	101
12.4	45.6	34.6	97
12.4	45.6	35.8	101
12.4	45.6	35.3	100
12.4	22.8	23.7	99
12.4	22.8	23.4	97

* Filter 520 used.

TABLE IV

Comparison of Colorimetric and Nitrous Acid Manometric Methods (on Tungstic Acid Filtrates of Whole Blood from Rats)

Source of sample	Colorimetric method, amino N	Nitrous acid method, amino N	Difference
	mg per cent	*mg per cent*	*per cent*
1. Normal rat	14 0*	14.0	0.0
2. " "	14.3*	14.0	+2.0
3. " " partially hydrolyzed casein added	17 0	16.6	+2.5
4. Normal rat; partially hydrolyzed casein added	15.6	15.7	−0.6
5. Normal rat; complete casein hydrolysate added	35.7	35.7	0.0
6. Rat subjected to hemorrhage	22.5	20.8	+8.0
7. Eviscerated rat	24.5*	24.1	+1 6
8. " "	29.2	29.0	+0.7
9. " "	33.5	33.9	−1.2
10. " "	32.6	34.0	−4.1
11. " "	38.4	40.6	−5.2
12. " "	39.8	41.4	−3.8
13. " "	48.5*	44.9	+8.0

* Filter 520 used.

of the casein hydrolysate was determined by the colorimetric method on aliquots of the solution added to the blood samples. The recovery of

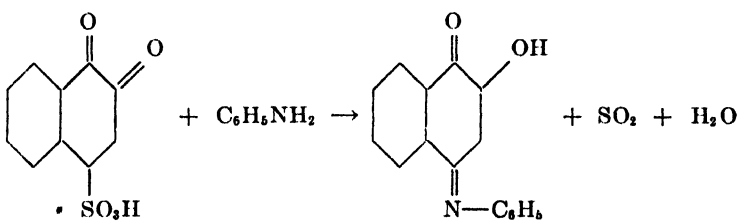
added amino acid in ten such experiments is given in Table III. The mean recovery was 99 per cent of that added.

Comparison of Colorimetric and Nitrous Acid Methods—Parallel determinations of the amino acid nitrogen content of tungstic acid filtrates of rat blood were made by the present colorimetric and Van Slyke manometric nitrous acid methods. The results are presented in Table IV. The agreement between the results of the two methods is fairly close, and there is no systematic discrepancy under these circumstances.

Amino Acid Nitrogen Content of Blood—In a series of twenty unanesthetized normal rats fasted 24 hours, the amino acid nitrogen content of whole blood ranged from 14.3 to 18.2 mg. per cent, with a mean of 16.6 ± 0.34[2] mg. per cent. In two other series of normal fasted rats, the amino acid nitrogen of blood plasma was 7.72 ± 0.49 mg. per cent and of serum, 11.33 ± 0.22 mg. per cent. In a few samples of human plasma, the average amino acid nitrogen content was 4.6 mg. per cent, and in eight human sera, 6.3 mg. per cent. A similar difference between the levels of amino acid nitrogen in plasma and in serum was observed by MacFadyen (7), who attributed it to release of amino acids during clotting.

DISCUSSION

The reaction of β-naphthoquinone sulfonate with amino acids and related substances is a remarkable one in that such a wide variety of structures leads to the formation of compounds whose colors are so similar both qualitatively and quantitatively. The reaction of the amino acids is believed to be analogous to the reaction of aniline with β-naphthoquinone sulfonate, which has long been known (8). The quantitative yield of



similar color with aniline under the special conditions of our analysis seems to confirm this belief. With only a few of the compounds other than amino acids which we tested was some variation encountered in the colors produced. Orthoform and the arsenicals, in which the amino group is ortho to a phenolic hydroxyl, produced compounds which were deep purplish red in alkaline solution; however, in acid these substances were the orange-red typical of amino acid-naphthoquinone compounds. Adrenalin,

[2] Standard error of the mean.

cobefrin (a primary amine containing, like adrenalin, a dihydroxyphenyl group), uric acid, and allantoin all yielded compounds which were much more red than the amino acid compounds. The reaction of the quinone with uric acid, which does not occur in the cold according to Folin, and with allantoin were surprising. None of the other compounds tested which contained similar structures, the purines, uracil (2,6-dioxypyrimidine), the imidazole ring in histidine, or urea, gave any color; so that the reactive group of uric acid and allantoin must be in the oxyimidazole ring. It would appear that the presence of a carbonyl group here activates one of the adjacent imino nitrogen atoms, perhaps by allowing the ring to be split at that point when attacked by the quinone reagent.

The colorimetric method described here has been so far applied only to the determination of amino acids in protein-free blood filtrates and to mixtures of amino acids. There appears to be no reason why it cannot be applied also to protein-free extracts of tissues and media from tissue metabolism experiments. Whether it can be used to determine amino nitrogen in such materials as partially hydrolyzed protein digests remains to be determined. Possible application of the method to the estimation of diamino acids is suggested by the quantitative yield of color of both amino groups in these compounds. Comparison of the results of the ninhydrin and colorimetric procedures on, for instance, a mixture of basic amino acids should give a measure of the amount of lysine present.

None of the available methods for estimating amino acid nitrogen in a mixture of amino acids gives identical results with all amino acids or is absolutely specific for amino nitrogen. The results of formol, alcohol, and acetone titrations vary with different amino acids, depending upon the choice of initial pH and other analytical conditions; they also are not specific for amino acids, being affected particularly by the presence of other organic acids (2). The nitrous acid reaction is a general one for primary amines, of which, of course, amino acids and their derivatives are the principal representatives in most biological material. Under the usual analytical conditions, nitrous acid reacts with 25 per cent of the ammonia nitrogen and 7 per cent of any urea nitrogen present. The prolines do not yield nitrogen gas with nitrous acid, the second amino group of lysine reacts slowly, and the results with glycine and cystine may be high. The ninhydrin gasometric (or titrimetric) procedure is the most specific, measuring free carboxyl nitrogen almost exclusively;[3] but it does require small corrections for urea and for proteins when these are present (7). The quantitative results of this reaction with different amino acids depend to a considerable extent upon the conditions chosen for analysis, and in

[3] This means that peptides do not ordinarily yield carbon dioxide; but glutathione gives one carboxyl per mole, equivalent to one-third of its total nitrogen.

any one set of circumstances used for the analysis of a mixture of amino acids, some compromise is necessary (9). At pH 2.5, recommended for the analysis of most complete mixtures of amino acids, aspartic acid yields two carboxyl groups for one nitrogen, lysine gives up somewhat more than the equivalent of one carboxyl per mole, and the results with tryptophane, cystine, and glycine are low. In the light of these considerations, it appears that the present colorimetric method is the equal of other available reactions for the quantitative estimation of amino nitrogen in a mixture of amino acids. The method is exceeded in specificity by the ninhydrin reaction, but considering the nature of substances likely to be present in most biological material, the specificity of the colorimetric procedure, like that of the nitrous acid method, is sufficient to make it practicable in most circumstances. The colorimetric method is of course not expected to replace the precise manometric techniques, but its greater sensitivity and simplicity recommend it as an additional method for estimating amino acid nitrogen.

SUMMARY

A convenient and sensitive procedure for estimating amino acid nitrogen has been described for use with a photoelectric colorimeter. The method is applicable to samples containing 4 to 40 γ of amino nitrogen and permits duplicate determinations to be made upon 0.2 ml. of blood. Recovery of amino acids added to blood is complete, and agreement of the results with those of the Van Slyke nitrous acid method is satisfactory.

The visible absorption spectra of the compounds formed by the reaction of the color reagent (β-naphthoquinone sulfonate) with seventeen amino acids were obtained.

Quantitative measurements were made of the reaction of twenty-one amino acids and of a number of other nitrogenous substances. The accuracy of the estimation of amino nitrogen in mixtures of amino acids and the specificity of the reaction are sufficient to allow application of the method to most biological material.

BIBLIOGRAPHY

1. Folin, O., *J. Biol. Chem.*, **51**, 377 (1922).
2. Van Slyke, D. D., and Kirk, E., *J. Biol. Chem.*, **102**, 651 (1933).
3. Danielson, I. S., *J. Biol. Chem.*, **101**, 505 (1933).
4. Sahyun, M., *J. Lab. and Clin. Med.*, **24**, 548 (1939).
5. Goodman, L., and Gilman, A., The pharmacological basis of therapeutics, New York, 959 (1941).
6. Shipley, R. A., and Long, C. N. H., *Biochem. J.*, **32**, 2242 (1938).
7. MacFadyen, D. A., *J. Biol. Chem.*, **145**, 387 (1942).
8. Gilman, H., Organic chemistry, New York, 878 (1938).
9. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).

THE VERATRINE ALKALOIDS

XIX. ON PROTOVERATRINE AND ITS ALKAMINE, PROTOVERINE

By WALTER A. JACOBS and LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 19, 1943)

Protoveratrine is a sparingly soluble alkaloid which comprises an appreciable fraction of the crystallizable alkaloids of *Veratrum album*. It was first isolated by Salzberger (1), who ascribed to it the formulation $C_{32}H_{51}O_{11}N$. This formulation has since been revised to $C_{40}H_{63}O_{14}N$ by Poethke (2), who succeeded in isolating from it on alkaline hydrolysis acetic acid, methylethylacetic acid, and methylethylglycolic acid. A supposed alkamine, *protoverine*, for which a corresponding formulation, $C_{28}H_{45}O_{10}N$, was assumed, was obtained with difficulty and only in amorphous form and no crystalline derivatives were prepared from it. Protoveratrine was thus concluded to be the triacyl ester of the alkamine, protoverine.

In our previous communication on the dehydrogenation of protoveratrine itself (3), we have described the production of 2-ethyl-5-methylpyridine, which had already been shown to be a characteristic basic dehydrogenation product from cevine (4) and jervine (5), and which more recently has been found to be a product also from rubijervine (6), germine (7), and from the potato alkamine, solanidine (8).[1] In addition, the formulations of jervine, rubijervine (and isorubijervine), and germine have been revised to $C_{27}H_{39}O_3N$, $C_{27}H_{43}O_2N$, and $C_{27}H_{43}O_3N$, respectively, so that, like cevine, $C_{27}H_{43}O_8N$, and solanidine, $C_{27}H_{43}ON$, they are all C_{27}

[1] Since the appearance of our paper (8) on the correlation of the veratrine alkaloids with the potato alkaloids, the recent article of Prelog and Szpilfogel (9) has reached us. These workers have also shown that the base which results on dehydrogenation of solanidine is 2-ethyl-5-methylpyridine. They have confirmed our earlier conclusions as to the identity of this base by its comparison with a base obtained by synthesis. They have adopted the same structural hexacyclic arrangement of the ring system for solanidine which we have already clearly expressed in the case of the veratrine alkaloids (10) with the exception of the possibility that Ring B can be 5-membered. Our discussion contained also the definite suggestion of a resemblance to the picture to be found in the case of the potato alkamine, in which, however, Ring B must be 6-membered as in the sterols, since Diels' hydrocarbon had been obtained from it on dehydrogenation. At that time, it was already our intention to test this suggested analogy by a similar study of the solanum alkamines, the results of which we have since published. We shall continue to regard the solanum alkamines as a subject of our studies, since they are members of the general group to which the veratrine alkaloids also belong.

derivatives. In view of the apparent discrepancy in the case of protoveratrine, we have returned to a study of this alkaloid.

It has now been found possible to isolate the alkamine, *protoverine*, in crystalline form by continuous extraction of the saponification mixture with chloroform. As will be presented further on, preliminary saturation of the diluted mixture with CO_2 was found necessary to reduce to a minimum isomerization to an *isoprotoverine*. Protoverine readily crystallized on concentration of the chloroform extract with chloroform of crystallization. This permitted its sharp separation from isoprotoverine, which remained in the mother liquor. Protoverine also crystallized from methanol and from water with solvent of crystallization. Analyses of the anhydrous substance have agreed with the formulation $C_{27}H_{43}O_9N$ and not with $C_{28}H_{45}O_{10}N$, as derived by Poethke. This formulation has been supported by the results obtained with a number of its derivatives. Attempts to hydrogenate protoverine with platinum oxide catalyst as the free base, or as the hydrochloride in methanol, were unsuccessful. However, on reduction with sodium in butanol, a *dihydro base*, $C_{27}H_{45}O_9N$, was obtained. The same dihydro base was obtained also from isoprotoverine described below. Whether it is to be regarded as a *dihydroprotoverine* or *dihydroisoprotoverine* has not as yet been determined.

Although the hydrochloride of protoverine could not be made to crystallize, the alkamine readily formed a crystalline *acetonylprotoverine hydrochloride*, which in turn yielded the crystalline *acetonylprotoverine*, $C_{30}H_{47}O_9N$. The formation of this derivative parallels that of acetonylgermine (7) and indicates that two of the hydroxyl groups in each of these alkamines are situated on 2 vicinal carbon atoms, or on carbon atoms removed by an intervening carbon atom. It is probable that these positions will be found to be identical in both of these alkamines.

The chloroform mother liquor of the above protoverine-chloroform compound after removal of solvent yielded material which crystallized from methanol as needles contaminated with prisms of protoverine, which were separated by fractionation. The needles were found to consist of *isoprotoverine*, $C_{27}H_{43}O_9N$. This substance was shown to be the result of the isomerizing action of alkali on protoverine, since the latter, when treated under certain conditions with alkali, was partly converted into isoprotoverine. If too strong alkali or too high a temperature was employed, the alkamine was further altered with the formation of a deep yellow solution, from which very little crystalline material could be isolated.

Contrary to protoverine, isoprotoverine could be hydrogenated to a *dihydroisoprotoverine*, $C_{27}H_{45}O_9N$, which was distinct from the product of the sodium reduction described above. This isomerism is probably due to the different position occupied by the double bond in each of these bases. As will be presented in a subsequent paper, a parallel picture is to

be found in the relationship between germine and isogermine. Although, as in the case of protoverine, isoprotoverine was found to react with acetone, no crystalline product could be obtained from the reaction mixture.

The formation of dihydro derivatives from protoverine, together with other observations, points definitely to the presence of a double bond in this alkamine. Like cevine, germine, and rubijervine, protoverine behaves as a tertiary base. The results of the active H determinations were in close agreement with the presence of nine hydroxyl groups in the alkamine. In support of this, acetonylprotoverine gave approximately 7 moles of methane as required by the presence of seven free OH groups. This conclusion was further supported by the result of the Zerewitinoff determination on the alkaloid protoveratrine itself. This approximated the requirements of seven free OH groups. Although the alkaloid must be a triacyl ester of protoverine, the extra OH group is contained in the α-hydroxymethylethylacetyl group. Since it contains nine hydroxyl groups and one double bond, protoverine, $C_{27}H_{43}O_9N$, like cevine, germine, and solanidine, must therefore be a hexacyclic tertiary sterol (or modified sterol) base.

As the triacyl ester of protoverine with acetic, methylethylacetic, and hydroxymethylethylacetic acids, the formulation of protoveratrine, therefore, must be $C_{39}H_{61}O_{12}N$. All of our attempts to obtain proper analytical results with the alkaloid after repeated recrystallization, however, have not been satisfying. It is possible that retention of solvent may have contributed to this result. Somewhat better analytical results were obtained with the alkaloid which had been recovered after exposure to the conditions for the production of an acetonyl derivative, such as that used in the case of its alkamine, protoverine. The failure to form such an acetonyl derivative shows that one or both of the hydroxyl groups responsible for this function in the alkamine must be covered by acylation in the alkaloid. It is probable that one of the acylated OH groups must be situated on carbon atom 3 of the sterol or modified sterol skeleton. This point will be discussed further in a subsequent paper.

EXPERIMENTAL

Protoveratrine—For the isolation of this alkaloid from the crude benzene extract of mixed alkaloids, we have followed essentially the procedure outlined in our previous paper (3). The analytical results from the material obtained by a repetition of our previous recrystallization procedure have been essentially duplicated. Again the substance separated on addition of ammonia to the solution of the acetate in alcohol as small four-sided platelets which decomposed at 275° (uncorrected)[2] after pre-

[2] The uncorrected melting points were taken in the usual manner, but not corrected for stem exposure. The others are corrected micro melting points.

liminary darkening and sintering. The decomposition point depended upon the rate of heating. When the substance was dried both at 120° (*a*) and 140° (*b*) at 2 mm., no appreciable loss could be noted.

$C_{39}H_{61}O_{13}N$.	Calculated.	C 62.28, H 8.18
	Found. (*a*)	" 61.48, " 7.95
	" (*b*)	" 61.76, " 7.72

This material was recrystallized several times by addition of ether to the chloroform solution. No retention of solvent could be detected on drying.

Found, C 61.28, H 8.11

Similar results were obtained when recrystallization was accomplished by addition of 95 per cent alcohol to the solution in chloroform, followed by boiling off the chloroform. The substance separated as six-sided platelets and prisms, which decomposed at 275° (uncorrected) after preliminary discoloration.

Found, C 61.48, H 7.98; C 61.56, H 7.94

3.720 mg. of substance gave 0.910 cc. of CH_4 (25°, 745.3 mm.); at 95° there was no change. Found, H 0.99; calculated for 7H, 0.94.

Protoveratrine was then treated with acetone and HCl under conditions which caused the production of an acetonyl derivative from protoverine as follows: Although the alkaloid itself was recovered, the analytical data approached more nearly the theoretical values.

0.1 gm. of protoveratrine was suspended in 2 cc. of acetone and continuously treated with HCl (1.19) until acid to Congo red and solution was complete. The mixture could not be made to crystallize, even on addition of ether. After dilution with water, the mixture was promptly made alkaline and extracted with ether. The latter on concentration gave the original alkaloid.

Found, C 61.91, H 8.02

In another experiment, the acidified acetone solution of the alkaloid was allowed to stand for 20 hours. It was treated directly with Na_2CO_3 solution, then diluted, and extracted with chloroform. The extracted material crystallized from ether as six-sided platelets.

Found, C 61.84, H 8.08

In an experiment in which the mixture stood for 3 days, similar results were obtained. The substance recrystallized from chloroform-ether decomposed at 280–283° (uncorrected) after preliminary darkening.

Found, C 61.87, 61.55, H 7.68, 8.16

Protoverine—3.8 gm. of protoveratrine were treated with 26 cc. of a solution of 4 gm. of NaOH in 100 cc. of methanol. Gentle warming caused rapid solution and the odor of fatty acid esters quickly became apparent. After about 7 minutes warming, the weakly colored solution was cooled, made just acid with dilute H_2SO_4, and then made faintly alkaline to phenolphthalein with Na_2CO_3 solution. The mixture after dilution to about 100 cc. was placed in a special continuous extractor for extraction with chloroform. The condensed chloroform, which fell in a continuous spray through the aqueous mixture to a lower chloroform layer, continuously passed through an overflow into the flask of boiling chloroform below. After about 20 hours, the extraction was interrupted. The flask contained a copious suspension of delicate needles. The collected material amounted to 1.8 gm.

For recrystallization, the chloroform compound was dissolved in a necessarily large volume of methanol and concentrated to 20 cc. The base separated as small, glistening, sparingly soluble prisms. The yield was 1.28 gm. It softened gradually to a slowly effervescing resin at 195–200°, after preliminary sintering. The melting point, however, varied with different preparations, which was due, apparently, to a varying solvent content.

$$[\alpha]_D^{25} = -12° \ (c = 0.96 \text{ in pyridine})$$

For analysis, the substance was dried at 120° and 2 mm.

$C_{27}H_{43}O_9N \cdot 2CH_3OH$.	Calculated,	CH_3OH 10.87; found, 9.92
$C_{27}H_{43}O_9N$.	"	C 61.67, H 8.25
	Found. (*a*)	" 61.45, " 8.30
	" (*b*)	" 62.06, " 8.32

A sample of the substance was dissolved by heating in a small volume of diluted methanol and, after addition of sufficient water, the methanol was boiled off. The base gradually crystallized as prismatic needles, which slowly softened to a melt at 210–216° (uncorrected). The aqueous solution foamed readily.

For analysis, the substance was dried at 120° and 2 mm.

$C_{27}H_{43}O_9N \cdot H_2O$. Calculated, H_2O 3.31; found, 3.22

Anhydrous Substance—Found, C 61.37, H 8.33.

3.685 mg. of substance gave 1.58 cc. of CH_4 (26°, 729.4 mm.); at 95° there was no change. Found, H 1.69; calculated for 9H, 1.73.

When the chloroform mother liquor of the above chloroform compound of protoverine was concentrated *in vacuo* to dryness, the resinous residue weighed 1.24 gm. When dissolved in a small volume of methanol, it readily crystallized on rubbing as minute platelets mixed with prisms and occasionally needles. After collection with methanol, 0.63 gm. was

obtained. On treatment with chloroform, this was found to yield an additional amount of the chloroform compound of protoverine. The mother liquor of this contained a small amount of isoprotoverine. If the diluted saponification mixture was not first made weakly alkaline, as described above, before the continuous extraction with chloroform, a larger proportion of isoprotoverine was obtained as described below under isoprotoverine.

Acetonylprotoverine—80 mg. of protoverine were suspended in 1 cc. of methanol and then brought into solution with sufficient HCl (1.19) to render the mixture distinctly acid to Congo red. On cautious addition of acetone to the initial precipitation point, the precipitate gradually redissolved, and it soon became possible to continue the addition of acetone without the formation of the amorphous material. On being rubbed, the hydrochloride of the acetonyl derivative crystallized in excellent yield. On recrystallization from methanol-acetone, it separated as microscopic aggregates of short, flat needles which melted with decomposition at 278–281° (uncorrected) after preliminary discoloration and sintering.

For analysis, the salt was dried at 110° and 2 mm.

$C_{30}H_{45}O_9NCl$. Calculated, C 59.82, H 8.04; found, C 59.62, H 8.10

The free base was readily obtained by addition of an excess of Na_2CO_3 solution to the solution of the salt in 50 per cent methanol. For recrystallization, a relatively large volume of methanol was required and, when concentrated to smaller volume, acetonylprotoverine separated as microscopic flat needles or elongated platelets. After initial sintering and coloring above 235°, it gradually melted to a dark mass at 253–256° (uncorrected).

$C_{30}H_{47}O_9N$. Calculated, C 63.67, H 8.38; found, C 63.58, H 8.45

4.797 mg. of substance gave 1.40 cc. of CH_4 (26°, 729.4 mm.); at 95° there was no change. Found, H 1.15; calculated for 7H, 1.25.

Reduction of Protoverine (Dihydroprotoverine?)—2 gm. of sodium were added to a boiling solution of 0.5 gm. of protoverine in 20 cc. of butanol, and the mixture was at once vigorously shaken. The reaction mixture colored when air gained access to it. After completion, the chilled mixture was treated with water and saturated with CO_2. The butanol was removed under reduced pressure, and the diluted mixture was continuously extracted with chloroform overnight. The accumulated chloroform extract contained crystalline material. After concentration to small volume, the crystals were collected with chloroform. The yield was 0.16 gm. For recrystallization, the product was dissolved in a necessarily large volume of methanol. After concentration to 10 cc., it separated without solvent

as micro platelets which, although mostly irregular, showed a tendency to be triangular. Under the microscope, it did not exhibit a real melting point. It began to show decomposition above 300°, which gradually increased in rate, especially at 330–335°.

$[\alpha]_D^{25} = -54°$ ($c = 0.50$ in pyridine)

$C_{27}H_{45}O_9N$.	Calculated.	C 61.44, H 8.60
	Found. (*a*)	" 61.34, " 8.53
	" (*b*)	" 61.52, " 8.61

Isoprotoverine—In a number of experiments in which the precaution was not taken to neutralize the saponification mixture before extraction, a larger proportion of this alkamine resulted. The mother liquors of the chloroform compound of protoverine, which had accumulated from the saponification of 15 gm. of protoveratrine, were concentrated to remove chloroform. The residue was dissolved in a small volume of methanol and then diluted with water and, after addition of Na_2CO_3 solution, the mixture was reextracted in a continuous extractor with chloroform for 20 hours. The chloroform extract on concentration yielded a slowly crystallizing fraction of needles, which proved to be isoprotoverine. The filtrate was concentrated and the residual chloroform was boiled off after addition of methanol. From a volume of about 20 cc. of methanol, a copious separation of needles occurred. This fraction of the isoalkamine, together with what crystallized directly from the above concentrated chloroform solution, amounted to 1.06 gm. The methanol mother liquor on further concentration formed a thick paste of a mixture of needles of isoprotoverine and rosettes of stouter crystals of protoverine.

Isoprotoverine separated on recrystallization from methanol as needles or thin, short prisms, which were practically without solvent. It began to color above 240°, then sintered and darkened on further heating, and effervesced at 264° (uncorrected).

$[\alpha]_D^{25} = -42°$ ($c = 0.99$ in pyridine)

$C_{27}H_{43}O_9N$.	Calculated.	C 61.67, H 8.25
	Found. (*a*)	" 61.54, " 8.34
	" (*b*)	" 61.70, " 8.37

Attempts to obtain a crystalline hydrochloride were unsuccessful.

The same substance was obtained directly from protoverine as follows: 0.2 gm. of protoverine was dissolved by warming in a mixture of 1 cc. of methanol and 3 cc. of H_2O. 1 cc. of N NaOH was added and the solution was warmed to 50° for 2 hours. After a few minutes, a turbidity developed which gradually increased to a deposit. The solution was treated with 5 cc. of methanol and diluted to about 80 cc. The mixture was extracted with chloroform for 18 hours in a continuous extractor. The addition

of methanol helped to reduce the tendency to form an emulsion during the extraction. The chloroform extract on concentration to a few cc. yielded a paste of needles which were collected with chloroform. 67 mg. were obtained. This consisted of unchanged protoverine. The mother liquor was concentrated to remove solvent and again dissolved in a little chloroform. A small second fraction was collected. Finally, a third small crop was obtained.

The final mother liquor was freed from chloroform by boiling down with methanol. The residue weighed 0.11 gm., representing roughly 50 per cent of transformed starting material. The solution in a small volume of methanol crystallized first as a mixture of pointed micro platelets, followed by a mass of delicate needles. The collected material weighed 53 mg. The homogeneity of the substance remained in question, since it did not dissolve completely in chloroform. By rapid fractionation from methanol, it was possible to remove first a rapidly crystallizing, small fraction of protoverine. This was followed by a fraction of needles of isoprotoverine.

The substance so obtained sintered above 200°, then gradually softened and darkened on further heating. The mass then melted with effervescence at 254° (uncorrected).

$$[\alpha]_D^{25} = -37° \ (c = 0.70 \text{ in pyridine})$$

For analysis, it was dried at 110° and 2 mm., since it was found to contain solvent.

$C_{27}H_{43}O_9N$. Calculated, C 61.67, H 8.25; found, C 61.63, H 8.25

As a check on identity, this substance was found to yield the following dihydro derivative.

Dihydroisoprotoverine—0.1 gm. of isoprotoverine (which had been obtained as a by-product on saponification of the alkaloid) was hydrogenated in methanol with 50 mg. of platinum oxide catalyst. The absorption was roughly 1 mole in excess of that required by the catalyst. During the hydrogenation, the substance which gradually dissolved was replaced by a suspension of the sparingly soluble product. A relatively large volume of a mixture of methanol and chloroform was required to redissolve the substance for separation from the catalyst. The filtrate, after concentration to remove the chloroform, yielded the substance as microscopic, short, flat needles or narrow platelets which contained solvent. In the micro melting point apparatus, it showed a slow decomposition at 315–320° after preliminary softening and discoloration. When treated under the microscope with a drop of water, it first dissolved and then crystallized again as delicate needles, apparently due to interchange of solvent.

$$[\alpha]_D^{25} = -49° \ (c = 0.67 \text{ in pyridine})$$

For analysis, the substance was dried at 120° and 2 mm.

$C_{27}H_{45}O_9N \cdot 2CH_3OH$. Calculated, CH_3OH 10.82; found, 10.61
Anhydrous Substance—$C_{27}H_{45}O_9N$. Calculated. C 61.44, H 8.60
Found. (*a*) " 61.29, " 8.63
" (*b*) " 61.40, " 8.50

Isoprotoverine, which was obtained by isomerization of protoverine, yielded on hydrogenation a substance indistinguishable in properties from the above.

Anhydrous Substance—Found, C 61.44, H 8.46.

All microanalyses and active H determinations were performed by Mr. D. Rigakos of this laboratory.

BIBLIOGRAPHY

1. Salzberger, G., *Arch. Pharm.*, **228**, 462 (1890).
2. Poethke, W., *Arch. Pharm.*, **275**, 357, 571 (1937).
3. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **143**, 427 (1942).
4. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **120**, 447 (1937); **124**, 659 (1938).
5. Jacobs, W. A., Craig, L. C., and Lavin, G. I., *J. Biol. Chem.*, **141**, 51 (1941).
6. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **148**, 41 (1943).
7. Craig, L. C., and Jacobs, W. A , *J. Biol. Chem.*, **148**, 57 (1943).
8. Craig, L. C., and Jacobs, W. A., *Science*, **97**, 122 (1943).
9. Prelog, V., and Szpilfogel, S., *Helv. chim. acta*, **25**, 1306 (1942).
10. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **141**, 253 (1941).

THE EFFECT OF PYRIDOXINE DEFICIENCY IN THE RAT UPON THE CATALASE ACTIVITY OF ITS TISSUES

By SAMUEL LEPKOVSKY and DELLA PARSONS

(From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley)

(Received for publication, May 10, 1943)

Fouts *et al.* (1) have shown that pyridoxine is essential for hemoglobin formation in the dog. This observation has been abundantly confirmed (2–4). Pyridoxine has also been shown to be essential for hemoglobin formation in the pig (5–7). There are fewer such data for the rat. The rat also seems to require pyridoxine for hemoglobin formation, though the anemia produced in pyridoxine-deficient rats does not seem to be as severe (8) as that produced in pyridoxine-deficient dogs and pigs.

Since pyridoxine is essential for the synthesis of hemoglobin, an iron-porphyrin complex, it seemed worth while to determine whether it was also essential for the synthesis of other iron-porphyrin complexes such as catalase, the cytochromes, and indophenol oxidase. The effect of pyridoxine deficiency on the catalase content of pyridoxine-deficient rats has been studied with two different groups of rats, and, since there was little difference between the groups, they are reported as one group.

EXPERIMENTAL

Care of Rats and Preparation of Factor 2 Concentrates—The rats were fed the pyridoxine-deficient diet previously described (9) plus 200 γ daily of synthetic calcium pantothenate.[1] The Factor 2 concentrates used were prepared as described (9) from rice bran extract (vitab rice bran concentrate, Nopco)[2] and from a liver fraction soluble in 93 per cent ethyl alcohol.[3] The equivalent of 0.16 gm. of liver fraction was fed daily.

The rice bran concentrate after treatment with fullers' earth still contained appreciable amounts of pyridoxine. This was removed by a single treatment with charcoal, 0.5 gm. of charcoal being used for each cc. of rice bran concentrate. 0.2 cc. of the concentrate was fed daily.

Physiological Food Control of Paired Fed Rats—Each pyridoxine-de-

[1] The synthetic vitamins used in this investigation were given to us by Merck and Company, Inc., Rahway, New Jersey.

[2] The vitab rice bran concentrate was given to us by The Vitab Corporation, Emeryville, California.

[3] The liver fraction was kindly furnished by Eli Lilly and Company, Indianapolis, Indiana.

ficient rat was controlled by a rat similarly fed and housed but getting 20 γ daily of pyridoxine. To obtain as exact a physiological food control as possible, correction had to be made for the increased efficiency in the utilization of food by the rat receiving pyridoxine (10). This was roughly accomplished by feeding the pyridoxine-fed rat 10 per cent less food than its deficient paired fed mate. As a result of this procedure the deficient and control rats in six pairs out of eleven were almost equal in weight. Of the remaining five pairs of rats, the efficiency in the utilization of food by the pyridoxine-fed rats was so much greater than that of their deficient paired fed mates that they were heavier even though they received 10 per cent less food. This difference in weight did not seem to affect the catalase activity of the rat tissues.

Catalase Determination and Preparation of Tissues—The method described by Balls and Hale (11) as modified by Bedford and Joslyn (12) was closely followed. The only change made was the introduction of 1 gm. of glucose directly into the reaction flask instead of in a solution of about 40 cc. of water (12). The volume was then made up with phosphate buffer to 49 cc., after which 1 cc. of 0.2 N hydrogen peroxide was introduced. This change was made on the suggestion of Dr. M. A. Joslyn to whom we are grateful for guidance in the catalase determination. The catalase determinations are reported as catalase activity per gm. of wet tissue (12).

A weighed portion of perfused tissue was very finely ground in a homogenizer (13) in 1 to 2 cc. of a phosphate buffer (12). The fibrous portions of the tissues were centrifuged out and the solution further diluted with phosphate buffer until the solution contained 1 gm. of wet tissue per 100 cc. of solution. Care was taken in the killing of the deficient rat and its paired fed control to keep all possible factors as uniform as possible, especially time, so that possible differences in catalase activity of the tissues of the controls and deficient rats would not be due to the technique in the determination of catalase.

Catalase Activity of Tissues of Normal Rats—In order to decide what tissues should be used for catalase determinations, a search was made in the literature for data on the catalase activity of normal rat tissues. Such data were scarce and fragmentary. This information was therefore obtained preliminary to the study of the catalase activity of the experimental rats. The tissues of two rats were analyzed and their catalase activity is given in Table I.

Catalase Activity of Pyridoxine-Deficient Rats—The tissues chosen for study were the liver, kidney, and heart and the results are shown in Table II.

The difference in catalase activity in the tissues of rats as a result of pyridoxine deficiency, although almost always in favor of the control, is not significant as judged by the t test. This is in contrast to the results ob-

TABLE I

Catalase Activity in Tissues of Normal Stock Rats (Female)

Age	Weight	Catalase activity								
		Liver	Kidney	Spleen	Lung	Fat	Brain	Adrenals	Muscle	R.b.c.
days	*gm.*									
240	291	148.0	49.80	5.56	6.70	1.15	0.38	3.72	0	46.50
201	203	166.7	54.31	6.51	7.52	1.02		4.60	0	48.00

TABLE II

Catalase Activity of Tissue of Experimental Rats

Pair No.	Group No.	Age	Treatment	Weight	Catalase activity		
					Liver	Kidney	Heart
		days		*gm.*			
1	3	395	Control	214	132.0	56.3	4.3
			Deficient	213	128.5	44 3	3.3
2	5	321	Control	158	129.5	56 6	4.9
			Deficient	163	128.7	49.5	4.1
3	6	390	Control	170	136.5	72.2	3.7
			Deficient	162	115.5	73.2	4.8
4	7	330	Control	130	137.0	47.5	4.3
			Deficient	122	123.0	59.4	3.6
5	8	392	Control	181	133.0	44.6	
			Deficient	161	127.0	39.8	
6	12	270	Control	186	141.0	54.6	4.6
			Deficient	155	142.8	57.2	4.5
7	14	306	Control	174	141.1	58.7	7.4
			Deficient	175	137.0	66.7	3.7
8	15	271	Control	194	125.0	42.5	5.0
			Deficient	195	138.1	38.5	4.7
9	16	141	Control	109	136.0	48.8	4.7
			Deficient	101	133.0	47.5	4.4
10	18	138	Control	110	140.0	46.5	4.0
			Deficient	107	138.0	51.0	4.4
11	21	61	Control	84	145.0	56.5	5.2
			Deficient	79	139.0	52.5	5.2
Average, control					136.0	53.2	4.8
" deficient					131.9	52.7	4.3
" difference					4.14	0.45	0.54
t value					1.428	0.18	1.233
P					>0.10	>0.80	>0.20

tained with copper- and iron-deficient rats (14) in which the catalase activity of the liver and kidney tissues was decidedly lower than that of the control rats.

CONCLUSION

Deficiency of pyridoxine does not cause any significant change in the catalase activity of the liver, kidney, and heart tissues of rats.

BIBLIOGRAPHY

1. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition*, **16**, 197 (1938).
2. Borson, H. J., and Mettier, S. R., *Proc. Soc. Exp. Biol. and Med.*, **43**, 429 (1940).
3. McKibbin, J. M., Schaefer, A. E., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, **142**, 77 (1942).
4. Street, H. R., Cowgill, G. R., and Zimmerman, H. M., *J. Nutrition*, **21**, 275 (1941).
5. Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *Biochem. J.*, **32**, 2207 (1938).
6. Wintrobe, M. M., Miller, M. H., Follis, H., Jr., Stein, H. J., Mushatt, C., and Humphreys, S., *J. Nutrition*, **24**, 345 (1942).
7. Hughes, E. H., and Squibb, R. L., *J. Animal Sc.*, **1**, 320 (1942).
8. Fouts, P. J., and Lepkovsky, S., *Proc. Soc. Exp. Biol. and Med.*, **50**, 221 (1942).
9. Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, **115**, 557 (1936).
10. Lepkovsky, S., in Evans, E. A., The biological action of the vitamins, Chicago (1942).
11. Balls, A. K., and Hale, W. S., *J. Assn. Off. Agric. Chem.*, **15**, 483 (1932).
12. Bedford, C. L., and Joslyn, M. A., *Ind. and Eng. Chem.*, **31**, 751 (1939).
13. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
14. Schultze, M. O., and Kuiken, K. A., *J. Biol. Chem.*, **137**, 727 (1941).

PRODUCTION OF THIAMINE DEFICIENCY DISEASE BY THE FEEDING OF A PYRIDINE ANALOGUE OF THIAMINE

By D. W. WOOLLEY and A. G. C. WHITE

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, May 6, 1943)

The production of bacterial growth inhibitors derived from acidic vitamins by the substitution of a sulfonic acid group for the carboxyl group of the vitamin is now well known. Thus sulfanilamide, the sulfur analogue of *p*-aminobenzoic acid (1), pyridine-3-sulfonic acid, the analogue of nicotinic acid (2), and thiopanic acid, the analogue of pantothenic acid (3–5), have been shown to prevent growth of certain microorganisms. Since addition of the analogous vitamin to cultures inhibited by one of these compounds restored growth, it seemed that the sulfonic acids caused failure of growth by producing deficiencies of the growth factors structurally related to them. However, these three sulfur analogues did not cause deficiency diseases when fed to animals (6, 7). The only deficiency diseases thus far produced in animals by the feeding of structurally similar compounds have been the hypoprothrombinemia caused by administration of 3,3′-methylenebis[4-hydroxycoumarin] (related to vitamin K) (8), and the scurvy-like disease caused by glucoascorbic acid.[1] Since a study of the action of these inhibitors related to the vitamins may provide a key to the study of the mode of action of the vitamins,[1] an attempt has been made to discover such inhibitors derived from some of the other vitamins.

The work of Erlenmeyer (9) suggested that physiological activity was partially retained in compounds in which sulfur in a ring system was interchanged with —CH:CH—. Others (10, 11) have endeavored to show that such an alteration in nicotinic acid (to yield thiazole-5-carboxylic acid) and in thiamine (to yield 2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl)pyridinium bromide) would result in compounds with vitamin action. However, Robbins (12) has shown recently that the latter substance, the pyridine analogue of thiamine, had no growth factor action, and actually inhibited growth of certain fungi.

It has now been observed that the feeding of 2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl)pyridinium bromide to mice maintained on an adequate diet caused a fatal disease, with many of the characteristic symptoms of thiamine deficiency as seen in other species. For the sake of brevity, the pyridine analogue of thiamine has been named

[1] Woolley, D. W., and Krampitz, L. O., unpublished data.

pyrithiamine. The disease produced by pyrithiamine administration was prevented or cured by the giving of sufficient amounts of thiamine. When mice were fed a ration free of thiamine, no characteristic symptoms, such as are seen in other species, were observed (13). The animals merely ate less and less, lost weight, and died. However, with administration of pyrithiamine, many characteristic symptoms of thiamine deficiency were seen in all animals. By variation of either the amount of thiamine or of pyrithiamine fed, it was found that about 40 molecules of pyrithiamine would nullify the effect of 1 molecule of thiamine. Hence, pyrithiamine was one of the most active vitamin antagonists thus far studied.

EXPERIMENTAL

Materials and Methods—Pyrithiamine was synthesized according to the directions of Tracy and Elderfield[2] (14). It was administered by giving 0.02 cc. of a solution of desired concentration orally three times daily. Each dose during the day was 4 hours removed from the previous one.

Weanling mice (approximately 12 gm.) were kept in individual cages equipped with screen bottoms and fed the following ration: sucrose 76 gm., vitamin-free casein 18 gm., salts (15) 5 gm., fortified corn oil (16) 1 gm., thiamine 80 γ, riboflavin 500 γ, nicotinic acid 10 mg., pyridoxine 200 γ, calcium pantothenate 2 mg., choline 10 mg., and inositol 100 mg. Such a ration was previously found adequate for mice (17). Each mouse ate about 2 gm. of this ration per day, and hence received 1.6 γ of thiamine. In the later experiments of this series, thiamine was omitted from the ration and administered orally once daily. The mice were weighed twice weekly.

Production of Disease—When sufficient pyrithiamine was given, the following sequence of events was observed in all animals with great regularity. Similar symptoms were seen to appear in every member of a group within 24 hours from the time the first mouse was affected. The animals failed to grow or lost weight, but there was no severe anorexia such as was seen in mice fed a thiamine-free ration. On the 4th or 5th day, they became very inactive and assumed a hunched posture. Then tremors and occasionally convulsions appeared, which were particularly marked when the animals were picked up by the tail. Somewhat later, spasticity, especially of the legs, appeared. Soon it was noted that when the mice attempted to stand erect on their hind legs, they would fall over backwards.

[2] A part of the 2-methyl-3-hydroxyethylpyridine used in the synthesis was supplied by Dr. M. Rubin of Wallace and Tiernan Products, Inc. The 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide used in the synthesis was donated by Dr. T. Jukes of Lederle Laboratories, Inc., and Dr. J. C. Keresztesy of Merck and Company, Inc. We wish to thank these men for their cooperation.

Violent jerking of the head backwards was also seen. Then the legs became weak and unable to support the body. This weakness always appeared on one side first and caused the animals to walk in circles. Following this, both sides became affected. Finally, all legs were affected to such an extent that the animals assumed a characteristic pose, lying on their bellies with the legs spread out on each side at right angles to the body. Shortly thereafter (within 24 hours) they died. No mouse survived longer than 3 days from the onset of symptoms. Data regarding responses to various dose levels of pyrithiamine are summarized in Table I. Except where otherwise stated, the experimental period was 2 weeks.

Cure and Prevention of Disease with Thiamine—A group of five mice was given 20 mg. of pyrithiamine daily until they had reached the stage at

TABLE I

Response of Mice to Various Doses of Thiamine and Pyrithiamine

Amount of pyrithiamine	No. of animals	Amount of thiamine	Time to produce symptoms	No. of animals showing symptoms	Average weekly weight change
mg per day		*γ per day*	*days*		*gm.*
0	35	1.6		0	+3.0
20	5	1.6	5	5	−3.0
2	12	1.6	7	12	−2.7
1.2	5	1.6	6	5	−2.1
0.6	5	1.6	8	5	−0.2
0.3	5	1.6	10	5	+1.9
0.1	5	1.6	11	5	+2.5
0.1	8	2	12	6	+2.4
0.05	3	2		0	+3.5
0.6	5	61.6		0	+3.1
2	8	60		0	+3.6

which they were unable to stand erect on their hind legs. The pyrithiamine treatment was continued and in addition one was given a single dose of 1 mg. of thiamine orally, two were given 500 γ each, and two were kept for controls. Each had lost 2.5 gm. in the 2 days prior to treatment. In all treated animals, improvement was noted within 1 hour, and within 20 hours they were without discernible symptoms. In the 24 hours following treatment, they had gained 1.5, 1.5, and 1.0 gm. The two which were not treated died within 2 days. No more thiamine was given to the treated animals, and within 5 days from the time of treatment they again developed symptoms and died.

Four mice were given 2 mg. daily of pyrithiamine until they were unable to stand. Two were then given 20 γ of thiamine each. The treated mice improved noticeably, but on the following day became worse again and soon died.

Prevention of the disease with thiamine was demonstrated in two experiments. In one, a group of eight mice received 2 mg. of pyrithiamine and 60 γ of thiamine per mouse per day. Appropriate control groups receiving no treatment and pyrithiamine alone were also included. These controls behaved similarly to comparable groups in the previous experiments; that is, mice in the group without pyrithiamine gained an average of 3.0 gm. per week and remained free of symptoms, while those in the group given pyrithiamine developed symptoms, lost weight, and all died within 9 days from the start of the experiment. In the second experiment, the mice received 600 γ of pyrithiamine and 61.6 γ of thiamine per day. Both experiments were continued for 17 days. The pertinent data are summarized in Table I. It can be seen that thiamine protected against the disease.

The effect of pyrithiamine was in some respects delayed and cumulative. Thus when three mice were given 1.2 mg. of pyrithiamine on each of the first 3 days of the experiment, and were then continued on 2 γ of thiamine per day, but without any more pyrithiamine, they grew well and appeared normal for 6 days. On the 7th day, they began to lose weight precipitously and to show the characteristic symptoms evoked by pyrithiamine. On the 9th day, one was treated with a single dose of 500 γ of thiamine. This produced remission of the symptoms within 24 hours. It was of interest that the symptoms appeared in this group almost as rapidly as in the group which had received 1.2 mg. of pyrithiamine every day (see Table I). Very small doses of pyrithiamine given daily for extended periods produced characteristic symptoms eventually. Thus in two experiments when eight mice were given 20 γ of pyrithiamine and 2 γ of thiamine per day, they grew as rapidly as controls which received no pyrithiamine, and remained without symptoms for $2\frac{1}{2}$ weeks. Following this, they ceased to grow, lost weight, and developed characteristic symptoms. These symptoms were promptly cured by a single dose of 1 mg. of thiamine. In a group of three mice which received 20 γ of pyrithiamine and 2 γ of thiamine per day for 14 days, and then 2 γ of thiamine a day but no pyrithiamine, symptoms developed at the 19th day and two of the mice died. The third mouse showed occasional violent fits of jumping, but slowly improved and survived for 4 weeks, at which time the experiment was discontinued.

The apparent delayed and cumulative effect of pyrithiamine made it difficult to establish an exact ratio of thiamine to pyrithiamine. From the curative tests it was seen that thiamine at one-fortieth the level of pyrithiamine would cure the disease. However, as little as 20 γ of pyrithiamine per day in the presence of 2 γ of thiamine per day eventually produced symptoms and death, even though growth was not affected in the early part of the period.

SUMMARY

The feeding of pyrithiamine, the pyridine analogue of thiamine (2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl)pyridinium bromide), to mice caused the appearance of characteristic symptoms of thiamine deficiency. The disease was prevented or cured by sufficient amounts of thiamine.

BIBLIOGRAPHY

1. Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).
2. McIlwain, H., *Brit. J. Exp. Path.*, **21**, 136 (1940).
3. Snell, E. E., *J. Biol. Chem.*, **139**, 975 (1941).
4. Kuhn, R., Wieland, T., and Moller, E. F., *Ber. chem. Ges.*, **74**, 1605 (1941).
5. McIlwain, H., *Biochem. J.*, **36**, 417 (1942).
6. Woolley, D. W., and White, A. G. C., *Proc. Soc. Exp. Biol. and Med.*, **52**, 106 (1943).
7. Martin, G. J., *Proc. Soc. Exp. Biol. and Med.*, **51**, 56 (1942).
8. Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, **23**, 589 (1942).
9. Erlenmeyer, H., *Helv. chim. acta*, **21**, 1013 (1938).
10. Schmelkes, F. C., *Science*, **90**, 113 (1939).
11. Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, **43**, 391 (1940).
12. Robbins, W. J., *Proc. Nat. Acad. Sc.*, **27**, 419 (1941).
13. Hauschildt, J. D., *Proc. Soc. Exp. Biol. and Med.*, **49**, 145 (1942).
14. Tracy, A. H., and Elderfield, R. C., *J. Org. Chem.*, **6**, 54 (1941).
15. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
16. Woolley, D. W., *J. Biol. Chem.*, **143**, 679 (1942).
17. Woolley, D. W., *Proc. Soc. Exp. Biol. and Med.*, **46**, 565 (1941).

THE EFFECT OF SIMULTANEOUS MINERAL AND CHOLINE DEFICIENCIES ON LIVER FAT

By PHILIP HANDLER

(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, May 14, 1943)

Fatty infiltration of the liver was not observed in rats fed a choline-deficient diet which simultaneously lacked thiamine (1), riboflavin (2), pantothenic acid (3), or methionine.[1] Fatty livers also failed to appear when rats were fed a choline-deficient diet containing sufficient nicotinamide to prevent growth or produce an actual loss in weight (4). It has been suggested (4, 5), in summary, that the characteristic fatty livers of choline deficiency can only occur when the composition of the remainder of the diet will permit at least moderate growth in young rats and nitrogen balance in adults. In a further test of this hypothesis the effects of a general mineral deficiency on the manifestations of choline deficiency have been studied.

EXPERIMENTAL

The basal diet used in this study consisted of casein 15, corn-starch 25, sucrose 20, lard 30, cod liver oil 5, and salts (6) 5. Each kilo of diet was supplemented with thiamine chloride 2.5 mg., pyridoxine hydrochloride 2.5 mg., riboflavin 5 mg., calcium pantothenate 30 mg., 3-methyl-1,4-naphthoquinone diacetate 1 mg., and choline 2.5 gm. When the salt mixture was omitted from the diet, an equivalent amount of starch was substituted. However, no such allowance was made for the small amount of choline.

Four groups of male rats were used in the following experiments. Group I received the basal diet, Group II received the basal diet without any salt mixture, Group III received the basal diet but no choline, and Group IV received the basal diet but neither choline nor salt mixture. Half of the rats in Groups I and III (Ib, Id, IIIb, and IIId) were pair-fed with the members of Group IV and the remainder offered food *ad libitum*. The results are summarized in Table I. The entire experiment was performed twice with six rats in each subgroup as it appears in Table I, so that each figure represents the mean value obtained for a group of twelve rats. All rats weighed between 90 and 100 gm. at the start of the experiment. The

[1] Handler, P., unpublished data.

variations within each group were surprisingly small and the differences reported in Table I are highly significant according to statistical analysis.

Despite the complete omission of salt mixture from the diets of Groups II and IV, the animals did grow, although slowly, for the first 2 weeks. In the following 17 day period all members of these two groups declined somewhat in weight.

The choline of the diets of Groups I and II afforded complete protection against fatty infiltration of the liver. The absolute fat content and size of the livers of Group IIIb which was pair-fed with Group IVa were not as great as those of Group IIIa which was fed *ad libitum*. However, the composition of the livers of Groups IIIa and IIIb was quite similar; both

TABLE I

Effect of Choline and Mineral Deficiencies on Liver Fat

Group No.	Diet	Time	Gain in weight		Food intake	Liver	Liver fatty acids	
		days	*gm.*	*gm. per day*	*gm. per day*	*gm*	*gm.*	*per cent wet weight*
Ia	Complete	14	22	1.5	8.6	5.29	0.212	4.0
Ib	"	14	18	1.25	7.8	4.50	0.189	4.1
IIa	No salts	14	15	1.1	7.7	4.77	0.181	3.8
IIIa	" choline	14	14	1.0	8.6	6.34	1.279	19.2
IIIb	" "	14	11	0.8	7.8	5.44	0.898	16.5
IVa	" salts or choline	14	13	0.9	7.8	5.20	0.582	11.2
Ic	Complete	31	54	1.7	8.8	5.37	0.228	4.1
Id	"	31	33	1.1	5.9	4.44	0.182	4.1
IIb	No salts	31	7	0.2	6.1	4.52	0.176	3.9
IIIc	" choline	31	59	1.9	9.4	8.54	1.988	21.2
IIId	" "	31	18	0.6	5.9	4.52	1.070	23.0
IVb	" salts or choline	31	3	0.1	5.9	4.12	0.230	5.6

groups were found to have decidedly fatty livers. At the same time, despite the general mineral deficiency, the slowly growing rats of Group IVa were found to have moderately fatty livers.

The second series of animals was sacrificed 17 days after the first series. Both subgroups on the complete ration had continued to grow during this period, while the salt-deficient animals in Group IIb had gradually declined in weight. However, no further change had occurred in the liver fat concentrations of the animals in these three groups. Restriction of the food consumption of Group IIId to the level seen in Group IVb still permitted slow growth. While the livers of the rats of Group IIIc, fed *ad libitum*, continued to increase in size and fat content, no further increase in size was seen in the livers of Group IIId, although there was some increase in

their fat content. Thus, the livers of Group IIId were only half as large as those of Group IIIc, although the fat concentration in the two groups was almost identical. Within this 17 day period the mineral- and choline-deficient rats of Group IVb lost about 10 gm. each. At the same time, there occurred a steady diminution in liver fat concentration and in the absolute amount of fat in the liver. This cannot be attributed to the poor appetite of these animals, since the rats in Group IIId showed an actual increase in liver fat and liver fat concentration during this period although their food consumption was limited to that of the members of Group IVb. This is quite similar to the results obtained earlier, when the effect of progressive thiamine deficiency on the liver fat of choline-deficient animals was studied (5).

The growth of choline-deficient rats has now been depressed by six independent techniques, thiamine, riboflavin, pantothenic acid, methionine, and mineral deficiencies and excessive nicotinamide feeding. In each instance the characteristic fatty livers of choline deficiency have failed to appear. These data would seem to warrant the conclusion that choline deficiency can only result in fatty liver formation when all other dietary factors are present in sufficient concentrations to permit at least slow growth.

SUMMARY

Young male rats fed a low protein, high fat diet deficient in choline and minerals grew slowly for 2 weeks and at the end of this time their livers were moderately fatty. In the following 2 weeks the animals declined in weight and the liver fat content returned towards normal. These effects were not due merely to the level of food consumption, since choline-deficient animals given adequate amounts of mineral salts but whose food consumption was restricted to that of the mineral-deficient animals continued to grow slowly and developed markedly fatty livers in the same period.

The author's thanks are due to the John and Mary R. Markle Foundation for its support of these studies and to Merck and Company, Inc., of Rahway, New Jersey, for the supply of crystalline vitamins used in this work.

BIBLIOGRAPHY

1. McHenry, E. W., *J. Physiol.*, **89**, 287 (1937).
2. Engel, R. W., *J. Nutrition*, **24**, 175 (1942).
3. McHenry, E. W., and Gavin, G., *Science*, **91**, 171 (1940).
4. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146**, 357 (1942).
5. Handler, P., and Bernheim, F., *J. Biol. Chem.*, **148**, 649 (1943).
6. Drummond, J. C., and Watson, A. F., *Analyst*, **47**, 235 (1922).

THE CHEMISTRY OF MOLD TISSUE

XVI. ISOLATION OF FUNGUS CEREBRIN FROM THE MYCELIUM OF ASPERGILLUS SYDOWI*

By NESTOR BOHONOS and W. H. PETERSON

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, February 12, 1943)

In the course of investigations (1–3) dealing with the nitrogen constituents of the mycelium of *Aspergillus sydowi*, an insoluble residue was obtained after autolysis and extraction with dilute sodium hydroxide. During the extraction of this residue with ether for several days, a precipitate gradually accumulated in the boiling flask. Repeated crystallization of the precipitate gave a product of constant melting point and apparently high molecular weight. From a study of its properties, derivatives, and degradation products, it appears to be identical with a compound first obtained by Zellner (4) from the mushroom, *Amanita muscaria*, which he named "fungus cerebrin." This compound has also been isolated from other mushrooms, *Hypholoma fasicular* (4, 5) and *Marasmius scorodonius* (6), from the bracket fungus, *Polyporus pinicola* (7), and from yeast (8–10). Reindel *et al.* (8, 9) worked over large quantities of extraction residues from the commercial production of ergosterol and obtained enough material for an extensive investigation covering nearly a decade of work. From their data they (9) deduced the structural formula,

$$C_{15}H_{31}\cdot CHOH\cdot CH(HN\cdot CO\cdot CHOH\cdot C_{24}H_{49})\cdot CHOH_2\cdot CH\cdot CH_2OH$$

The data that have been obtained for the crystalline material from *Aspergillus sydowi* agree well with those reported by Reindel and associates for fungus cerebrin, and thus add another material, mold mycelium, to the sources of this compound.

EXPERIMENTAL

Isolation—The mold was grown and autolyzed as described in a previous paper (1). The insoluble residue after autolysis was extracted several times with 1 per cent sodium hydroxide (3), washed first with 1 per cent

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

acetic acid, then with water, and finally dried. The product was ground to a very fine powder and extracted with ether in a Soxhlet extractor. During the extraction a white precipitate formed in the boiling ether and increased in quantity for many days. (With a Soxhlet apparatus of 1 liter capacity 1 month was generally required for complete extraction.) The precipitate was filtered off and washed with a little ether to remove ergosterol and fatty acids. The yields of this crude product (m.p. 118–132°) varied from 0.1 to 0.4 per cent of the dry mold. It was partially purified by several recrystallizations from alcohol.

When alkali-extracted unautolyzed mold was extracted with ether, there was no precipitate in the ether. This indicates that the compound is bound to other components of the mycelium and is set free by autolysis.

Acetylation—2 gm. of the crystals just described, 7 ml. of acetic anhydride, and 1 ml. of pyridine were heated on a steam bath for 2 hours. The excess acetic anhydride was decomposed with water in the usual manner, and the precipitate which formed was filtered, washed with water, and dried. The product was dissolved in ether, the solution was filtered, and the ether removed under reduced pressure. The acetate was recrystallized ten times from methanol before a constant melting point of 67.0–67.5° was obtained. (Reindel reported a melting point of 67–68°.) Yield 1.6 gm. This acetate is very soluble in benzene, ether, and chloroform, and is slightly soluble in ethyl and methyl alcohols. The specific rotation, composition, and molecular weight were as follows:

$[\alpha]_D^{23} = +20.6°$ ($\alpha = +1.89°$, $l = 2$ dm., $c = 4.6$, $CHCl_3$, 23°)
$C_{54}H_{101}O_9N$. Calculated. C 71.44, H 11.13, N 1.54
Found. " 71.05, 70.89; H 11.14, 11.24; N 1.60

Molecular Weight—0.444 and 0.971 gm. of compound in 13.04 gm. of benzene raised the boiling point 0.189° and 0.413° respectively. Mol. wt., found, 923, 921; calculated for $C_{54}H_{101}O_9N$, 907

Hydrolysis of Acetate—10 ml. of 0.3877 N alcoholic KOH and 5 ml. of ethanol were added to 0.545, 0.497, and 0.505 gm. samples of the acetate and refluxed for ½ hour. The alcohol was removed *in vacuo*, and CO_2-free water was added. The contents of the flasks were titrated to an end-point with phenolphthalein, stoppered, allowed to stand overnight in order to permit any occluded alkali to diffuse out, and titrated again. The total amounts of alkali required for hydrolysis of the three samples were 6.58, 5.96, and 6.14 ml. respectively. The corresponding saponification equivalents were 214, 215, and 212. Calculation for $C_{54}H_{101}O_9N$ as a tetraacetate equals 226.

Attempts to hydrolyze only part of the acetyl groups on the molecule were unsuccessful. Treatment of the acetate in a 1:1 ether-alcohol mixture with NaOH equivalent to one-fifth of the acetyl groups in the sample

resulted in complete hydrolysis of the compound and separation of the free compound in the form of fine needles. Upon recrystallization from alcohol the product melted at 142.5–143°. Reindel gives the melting point of fungus cerebrin as 143–143.5°. Purification through the acetate proved to be the best method for preparing the pure compound. The specific rotation and composition were as follows:

$[\alpha]_D^{22} = +11.9°$ ($\alpha = +0.24°$, $l = 2$ dm., $c = 1.01$, pyridine, 22°)
$C_{46}H_{93}O_8N$. Calculated. C 74.69, H 12.72, N 1.89
Found. " 74.92, 74.63; H 12.73, 13.13; N 1.92

Chemical Properties—The compound was only slightly soluble in methanol, ethanol, chloroform, carbon tetrachloride, acetone, ether, or benzene at room temperature but it was quite soluble in pyridine.

The compound was degraded when heated at 235°. Water was driven off, leaving a product that was very soluble in ether and chloroform but insoluble in methanol. On purification from a mixture of chloroform and methanol, the product softened at 71° and melted at 74–75°. It gave a positive test for unsaturation with tetranitromethane and took up bromine from a chloroform solution. Neither the free compound nor its acetate gave these tests. As this product appeared to have no advantage over the free compound for purposes of characterization, it was not investigated further.

When HCl gas was passed through a cold suspension of the compound in acetone, it dissolved very readily. The conditions of this experiment are suitable for formation of isopropylidene derivatives. As we already knew there were several hydroxyl groups, it appeared possible that some were on adjacent carbon atoms. This possibility was confirmed when a test was made with lead tetraacetate.

The compound was labile to refluxing in alcoholic N HCl, and this property was used in the later degradation studies.

Oxidation with Lead Tetraacetate—1 gm. of finely ground compound was dissolved in 75 ml. of dry chloroform at 40–50°. A solution of 0.6 gm. of lead tetraacetate in 20 ml. of dry chloroform and 0.5 ml. of acetic acid was added over a period of 30 minutes. The solution was kept at 40–50° and stirred continually during the addition. After it was held at 40–50° for another half hour, the lead acetate which had precipitated was filtered off, and the filtrate was concentrated to dryness *in vacuo*. The residue was extracted three times with 10 ml. volumes of petroleum ether (b.p. 20–40°). The petroleum ether-soluble fraction will be referred to as Fraction A, and the petroleum ether-insoluble fraction will be referred to as Fraction B.

The petroleum ether was removed from Fraction A, leaving 0.4 gm. of waxy residue with a strong paraffin-like odor. This melted at approx-

imately 30°. A 100 mg. aliquot in 2.5 ml. of ethanol was added to a suspension of 50 mg. of 2,4-dinitrophenylhydrazine in 7.5 ml. of ethanol. This was refluxed for 2 minutes, 0.1 ml. of concentrated hydrochloric acid was added, and refluxing was continued for 15 minutes. 2.5 ml. of water were added and the solution became cloudy. On cooling, a deposit of fine needles was obtained. These were filtered and washed with 50 per cent alcohol. After three recrystallizations from alcohol the orange-colored dinitrophenylhydrazone melted at 94–95°. (Reindel reported a melting point of 93.5–94°.) Yield 90 mg.

$C_{22}H_{36}N_4O_4$. Calculated. C 62.86, H 8.57, N 13.33
Found. " 63.14, " 8.39, " 13.34, 13.38

This corresponds to a carbonyl compound of formula $C_{16}H_{32}O$ and it is apparently the same as that isolated by Reindel *et al.* The remainder of Fraction A, a waxy solid, gradually increased its melting point on standing. This was no doubt due to the formation of a trimer as reported by Reindel.

Acid Hydrolysis of Fraction B—The petroleum ether-insoluble fraction from the lead tetraacetate oxidation was washed with water and dissolved in a mixture of 90 ml. of methanol and 10 ml. of concentrated HCl and refluxed for 6 hours. When this was cooled, the precipitate was filtered and washed with methanol-HCl. After two recrystallizations from methanol, this ester melted at 68–71°. Yield 0.44 gm. This was dissolved in a small amount of petroleum ether, filtered, and the solvent was removed *in vacuo*. A solution of this compound in 35 ml. of ethanol containing 4 ml. of 0.35 N alcoholic KOH was refluxed for 1 hour. After cooling, the solid that separated was filtered off and washed with alcohol and ether. It was dissolved in 15 ml. of hot acetic acid and on cooling the free acid separated. After several recrystallizations, the acid melted at 102.5–104.5°. Reindel *et al.* report the melting point of a corresponding product derived from cerebrin as 103–105°.

$C_{26}H_{52}O_3$. Calculated. C 75.72, H 12.62
Found. " 75.22, 75.19; H 12.52, 12.27

The ammonium salt was prepared by passing ammonia gas through an ether solution of the acid and analyzed.

$C_{26}H_{55}O_3N$. Calculated, N 3.19; found, N 3.17

When 50 mg. of the acid were heated in a sealed vial with 100 mg. of anhydrous chloral at 130° for 4 hours, a chloralide was formed. The excess chloral was removed with water and the residue was recrystallized from acetone and acetic acid. After many recrystallizations, the melting point was 65–66°. The quantity of material was too small for further recrystallization. Reindel (8) reports a melting point of 67–68° for his derivative.

50 mg. of the acid were dissolved in 5 ml. of glacial acetic acid and heated

on the steam bath. An equal weight of lead tetraacetate in 2 ml. of acetic acid was added in small portions over a period of 1 hour. The solution was heated for an additional half hour and then most of the acetic acid was removed *in vacuo* and water was added. The insoluble material was extracted with acetone. The acetone was removed and the semicarbazone of the aldehyde was formed after 1½ hours of refluxing in methanol with 50 mg. of semicarbazide hydrochloride and the appropriate amount of potassium hydroxide. The semicarbazone was recrystallized many times from methanol and ethyl acetate. After the sample had become too small for further purification, its melting point was found to be 113.5–114°. Reindel *et al.* give the melting point of their semicarbazide as 115–115.5°. Although these last two derivatives were not further purified and analyzed, the data indicate that we had the same hydroxy acid as was reported by Reindel *et al.*

The filtrate from the acid hydrolysis of Fraction B after removal of the ester of the hydroxy acid was concentrated to a small volume and taken up in water. After it was refluxed with 2,4-dinitrophenylhydrazine, in alcoholic solution, a very small amount of an orange-brown compound was formed. This decomposed at 270–280° but was not purified. It is probably the same as the dinitrophenylhydrazone of $C_4H_8O_3$ reported by Reindel *et al.*

SUMMARY

A crystalline lipid, believed to be identical with fungus cerebrin, has been isolated from the mycelium of *Aspergillus sydowi*, in yields of from 0.1 to 0.4 per cent of the dry mold tissue. The lipid was obtained by ether extraction of the insoluble material that remains after autolysis and alkali extraction of the mycelium. The compound appears to be bound in the mold tissue, as it could not be obtained from unautolyzed mycelium.

After repeated crystallization of the compound, a study was made of its properties, acetyl derivative, and degradation products. The analytical data on the free compound and its tetraacetate denote the formula $C_{46}H_{93}O_5N$. Cleavage of the molecule with lead tetraacetate produced a carbonyl compound, $C_{16}H_{32}O$, and a product which on hydrolysis yielded a hydroxy acid $C_{26}H_{53}O_3$. Also some evidence of the existence of a third decomposition product, possibly $C_4H_8O_3$, was obtained. These data conform to the structure $C_{15}H_{31} \cdot CHOH \cdot CH \cdot (CHOH \cdot CH_2 \cdot CH_2OH) \cdot NH \cdot CO \cdot CHOH \cdot C_{24}H_{49}$ proposed by Reindel *et al.* (9) for fungus cerebrin.

BIBLIOGRAPHY

1. Woolley, D. W., and Peterson, W. H., *J. Biol. Chem.*, **121**, 507 (1937).
2. Woolley, D. W., and Peterson, W. H., *J. Biol. Chem.*, **122**, 213 (1937–38).
3. Bohonos, N., Woolley, D. W., and Peterson, W. H., *Arch. Biochem.*, **1**, 319 (1942).

4. Zellner, J., *Monatsh. Chem.*, **32**, 133 (1911).
5. Rosenthal, R., *Monatsh. Chem.*, **43**, 237 (1922).
6. Fröschl, N., and Zellner, J., *Monatsh. Chem.*, **50**, 201 (1928).
7. Hartmann, E., and Zellner, J., *Monatsh. Chem.*, **50**, 193 (1928).
8. Reindel, F., *Ann. Chem.*, **480**, 76 (1930).
9. Reindel, F., Weickmann, A., Picard, S., Luber, K., and Turula, P., *Ann. Chem.*, **544**, 116 (1940).
10. Ruppol, E., *Bull. Soc. chim. biol.*, **19**, 1164 (1937).

LETTERS TO THE EDITORS

CHEMICAL AND BIOLOGICAL ASSAYS OF THE NICOTINAMIDE-LIKE SUBSTANCE FORMED IN HEATED MIXTURES OF ASPARAGINE AND GLUTAMIC ACID

Sirs:

It has been reported previously that heated mixtures of asparagine and glutamic acid can replace nicotinamide as a growth factor for certain microorganisms,[1] but no evidence as to the chemical nature of the reaction product could be obtained because of the very small quantities formed. It is now possible to produce this substance in concentrations sufficiently high to demonstrate that chemically as well as biologically it shows properties of nicotinamide.

A neutral solution containing 10 gm. each of asparagine and glutamic acid, 20 mg. of $FeSO_4 \cdot 7H_2O$, and 30 mg. of $MnSO_4 \cdot 4H_2O$ in a volume of 200 ml. was heated for 8 days in a stream of oxygen at 100°. The solution was then extracted with ether for 48 hours. The extracted material, after removal of the ether, was dissolved in 15 ml. of 0.05 M phosphate buffer, pH 7, and reextracted with ether. The second extract was dried and dissolved in 17 ml. of water. It contained over 90 per cent of the original growth-promoting activity and is referred to as Solution 190C.

Certain other amino acids can substitute for glutamic acid in the production of nicotinamide activity; the most active of these is methionine.[2] Therefore, a similar experiment was carried out with 10 gm. each of methionine and asparagine, except that the two were heated in a stream of air for only 5 days. The water solution of the material obtained from the final ether extract, Solution 270C, had a volume of 8.5 ml.

The two solutions were analyzed for nicotinamide biologically with *Lactobacillus arabinosus* by the previously described modification[1] of the method of Snell and Wright,[3] and chemically by measurement of the color developed with cyanogen bromide and aniline.[4] Chemical analyses were made both before and after hydrolysis. Nicotinamide was used as standard in the former case, nicotinic acid in the latter. The results are given in the table. Since the chemical method has shown a reproducibility of ±3 per cent and the biological of ±5 per cent, the three values for each solution must be considered to be essentially the same.

[1] Bovarnick, M. R., *J. Biol. Chem.*, **148**, 151 (1943).
[2] Bovarnick, M. R., unpublished observations.
[3] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
[4] Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, **134**, 1 (1940).

While the chemical method is not specific, different pyridine compounds do give varying amounts of color in the cyanogen bromide-aniline reaction. Even the change from nicotinic acid to nicotinamide produces a nearly 3-fold reduction in color intensity per unit of weight. Therefore, the good quantitative agreement between the three assays on the solutions obtained

Comparison of Chemical and Biological Analyses for Nicotinamide

Solution No.	Nicotinamide		
	Bioassay with *L arabinosus*	Chemical assay (nicotinamide as standard)	
		Before hydrolysis	After hydrolysis
	mg. per ml.	*mg per ml.*	*mg. per ml.*
190C. From asparagine-glutamic acid reaction	0.90	0.91	0.92
270C. From asparagine-methionine reaction	0.82	0.84	0.83

from both reactions would seem to be strong evidence in favor of the assumption that nicotinamide is the active compound formed. This is further strengthened by the well known biological specificity of nicotinamide and of nicotinic acid.[5]

Division of Laboratories and Research
New York State Department of Health
Albany

MARIANNA R. BOVARNICK

Received for publication, June 4, 1943

[5] Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Infect. Dis.*, **65**, 163 (1939).

AN IMMUNOLOGICALLY ACTIVE POLYSACCHARIDE PRODUCED BY COCCIDIOIDES IMMITIS RIXFORD AND GILCHRIST

By W. Z. HASSID, E. E. BAKER, and R. M. McCREADY*

(From the Division of Plant Nutrition of the College of Agriculture, University of California, Berkeley, and the Department of Public Health and Preventive Medicine, Stanford University School of Medicine, San Francisco)

(Received for publication, April 28, 1943)

The fungus *Coccidioides immitis*, the causative agent of coccidioidomycosis in man and certain wild and domestic animals, produces a substance which elicits a skin reaction when injected intradermally into sensitive individuals, and precipitates and fixes complement in the presence of serum of persons with coccidioidomycosis. The only data on the nature of this substance are found in the work of Hirsch and D'Andrea (1). These authors have shown that *Coccidioides immitis* produces a "specific soluble substance" which is precipitated by alcohol, contains 3 to 4 per cent nitrogen, and upon hydrolysis produces 20 to 40 per cent reducing sugars. The osazone prepared from the hydrolyzed solution was identified as glucosazone.

A filtrate of old cultures of *Coccidioides immitis*, known as coccidioidin, is widely used as an aid in the diagnosis of coccidioidomycosis (2). The present writers, therefore, found it of interest to study the "specific soluble substance" produced by this fungus and to ascertain its nature and whether a relation exists between its chemical constitution and its immunological properties.

EXPERIMENTAL

Preparation of the Polysaccharide—*Coccidioides immitis* (Strain 46) was grown for 2 months at 37° on a modified Bureau of Animal Industry tuberculin medium which had the following composition: *l*-asparagine 7.0 gm., NH_4Cl 7.0 gm., K_2HPO_4 1.3 gm., sodium citrate 0.5 gm., $MgSO_4$ 1.5 gm., ferric citrate 0.3 gm., cerelose 10.0 gm., glycerol 25.0 gm., distilled water to make 1 liter.

At the end of the incubation period, merthiolate was added to each culture flask to give a final concentration of 1 part in 10,000. The mat of fungus was then removed by filtration.

Lot 9 of the polysaccharide was prepared as follows: 4600 cc. of culture filtrate were concentrated by means of the collodion ultrafilters described

* Present address, Western Regional Research Laboratory, Albany, California.

by Seibert (3). Henderson[1] showed that the material responsible for eliciting a positive skin reaction in sensitive individuals does not pass through these filters. Thus, ultrafiltration offered a convenient method of concentrating this skin-reacting substance. After the filtrate had been concentrated to about 1 liter, this volume was maintained by the gradual addition of 2 liters of distilled water, followed by 3 liters of 1 per cent sulfuric acid. The dilute sulfuric acid was used to convert any acid groups present in the polysaccharide as salts to their free acids. Preliminary experiments had shown that certain dark brown impurities could be removed by fractional precipitation of the polysaccharide, containing free acid groups, with alcohol. Its salt, however, could not be purified in this manner. After addition of the dilute sulfuric acid, the residue was washed with 3 liters of distilled water and concentrated to 46 cc. The concentrate was free of salts, as shown by the absence of the sulfate ion.

The concentrate was then precipitated with 5 volumes of ethyl alcohol, and the precipitate collected by centrifugation and dissolved in 15 cc. of water. When 10 cc. of alcohol were added, a dark brown precipitate formed. This was removed by centrifugation (Fraction I). Upon the addition of 10 cc. of alcohol to the almost colorless supernatant solution, a white precipitate was obtained (Fraction II). This fraction was washed with 60 per cent alcohol, then with alcohol and ether, and dried *in vacuo* at 80°. Both fractions contained material which elicited positive tests in sensitive individuals. Although Fraction I, weighing 0.3 gm., showed some activity in skin tests, it was discarded. Fraction II weighed 1.7 gm. and was used in the subsequent work.

Properties of the Polysaccharide—The white amorphous precipitate obtained was soluble in water and distinctly acidic in aqueous solution. Its acid number was 3000 when neutralized to pH 8. Its ash content was 0.42 per cent; it contained 3.23 per cent total nitrogen and 0.6 per cent amino acid nitrogen, determined by the Van Slyke method (4). No acetyl groups were found in the carbohydrate. When oxidized with ferricyanide and titrated with ceric sulfate, the substance gave a reducing value of 9.5 per cent calculated as glucose (5). The polysaccharide gave a strong naphthoresorcinol test (6), indicating the presence of uronic acid in the molecule. The uronic acid content determined by adaptation of Dore's method (7) to a semimicro scale[2] was 10.5 per cent. Its carbon content was 43.8 per cent[3] (ash-free basis), and the specific rotation $[\alpha]_D$ +37.5°.

[1] Henderson, H. J., Henry Phipps Institute, University of Pennsylvania, unpublished data.

[2] Unpublished data.

[3] The carbon analyses in this investigation were carried out by the wet combustion method (McCready, R. M., and Hassid, W. Z., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 525 (1942)).

The Millon, xanthoproteic, glyoxylic acid, and biuret tests for proteins were all negative. Neither was any precipitate obtained by the addition of 5 per cent trichloroacetic acid to an equal volume of a 2 per cent polysaccharide solution, when the mixture was heated. This shows that no protein material was present in the carbohydrate.

Hydrolysis of the Polysaccharide and Identification of Products—When a 1 per cent solution of the polysaccharide was hydrolyzed by boiling with 1 N sulfuric acid, the liberation of the reducing sugars proceeded very rapidly. Within 30 minutes 27.5 per cent of the reducing sugars, calculated as glucose and determined by oxidation with ferricyanide and titration with ceric sulfate (5), was liberated. After 75 minutes, 79.2 per cent was present in the solution, and after 150 minutes the value diminished to 76.6 per cent. In connection with the diminution of the reducing value, Link and Nedden (8) showed that galacturonic acid is partly destroyed by prolonged heating with acid. This is probably due to decarboxylation of some of the uronic acid.

An osazone was prepared from the neutralized solution which was identified as glucosazone. The presence of glucose was later confirmed by the preparation of potassium acid saccharate, when a portion of the hydrolyzed solution was oxidized with nitric acid and neutralized with potassium carbonate. The solution gave a negative Seliwanoff reaction, indicating the absence of fructose. The aniline reaction (9), which distinguishes pentoses from uronic acids, was also negative, showing the absence of pentose sugars. The possibility of the presence of mannose was eliminated by the fact that no mannose phenylhydrazone could be obtained. Glucosamine, determined by the Elson and Morgan method (10), was shown to be absent.

Separation of Uronic Acid Constituent—A 1 gm. sample of the carbohydrate was dissolved in 10 cc. of water, an equal volume of 2 N sulfuric acid was added, and the solution boiled for 1.5 hours under a reflux condenser. It was then neutralized with barium carbonate, and the precipitate washed and removed by repeated centrifugation. The filtrate and washings were concentrated to about 2 cc. and the barium salt of the uronic acid precipitated by the addition of 10 volumes of methyl alcohol. The precipitate was centrifuged and washed by repeated centrifugation with methyl alcohol. After drying *in vacuo* at 60°, a yield of 0.150 gm. of the barium salt, the equivalent of 11 per cent uronic acid, was obtained. The methyl alcoholic filtrate and washings were combined and saved for further analysis of soluble sugars.

The barium salt gave the naphthoresorcinol test for uronic acid, and oxidation with nitric acid produced crystals of mucic acid. This showed that the salt was barium galacturonate.

Analysis—$(C_{12}H_{15}O_{14})Ba$. Calculated. C 27.5, Ba 26.3
Found. " 27.9, " 25.8

On treatment with phenylhydrazine hydrochloride and sodium acetate, a derivative was obtained from this salt which was identical with the phenylhydrazine phenylhydrazone of galacturonate described by Niemann, Schoeffel, and Link (11).

Analysis—$C_{18}H_{24}O_6N_4$. Calculated. C 55.1, N 14.3
Found. " 55.7, " 14.4

Identification of Glucose Constituent—The filtrate obtained after precipitation of the barium galacturonate was evaporated to a small volume to remove the methyl alcohol, quantitatively transferred into a 100 cc. volumetric flask, and diluted to volume. When analyzed by the ferricyanide method (5), the solution contained 0.720 gm. of reducing sugars, calculated as glucose. The specific rotation $[\alpha]_D$ of the solution, calculated on the basis of 0.720 gm., was $-7°$.

A portion of the solution, containing 0.140 gm. of reducing sugars, was concentrated to 2 cc. and oxidized with 2 cc. of nitric acid by evaporating the mixture on a watch-glass on the steam bath. The syrup was dissolved in about 1 cc. of water, made alkaline by the addition of potassium carbonate, and acidified with acetic acid. After refrigeration overnight, crystals were filtered from the solution and were identified as potassium acid saccharate by their appearance under the microscope (12) and by analysis of the potassium content.

The fact that the specific rotation of the solution was negative indicates that, together with the dextrorotatory glucose, there must exist another compound which is levorotatory. When a portion of the solution was heated in boiling water with phenylhydrazine hydrochloride and sodium acetate for 15 minutes and then cooled and examined under the microscope, two distinctly different osazones could be observed. A preponderance of glucosazone was recognized by the shape of its characteristic greenish yellow needle-like bundles (12). Mixed with this, in smaller proportion, was another osazone which had the appearance of dark green burs. This osazone has not yet been identified. When the solution was filtered and the filtrate heated for 30 minutes, and then cooled, more of this unidentified osazone crystallized.

In order to establish the relative proportions of glucose and the unknown sugar, a 10 cc. portion of the hydrolyzed solution, containing 72 mg. of reducing sugars, was inoculated with a pure culture of yeast, *Torula monosa*. A 0.5 per cent yeast infusion and 0.1 cc. of phosphate buffer of pH 6 were added and allowed to remain at 28° for 30 hours. At the end of this period the fermented solution contained 22 mg. of reducing sugars, calculated as glucose. On this basis, 50 mg., or 69.5 per cent, of glucose were present in the solution before fermentation. The remaining 22 mg. of reducing sugar

(30.5 per cent), obtained after fermentation of the glucose with *Torula monosa*, were therefore due to the unknown sugar. A blank, simultaneously run by inoculating 10 cc. of pure glucose solution (72 mg.) with *Torula monosa*, under identical conditions gave a reducing value after 30 hours equivalent to 0.6 mg. of glucose, showing that the yeast ferments glucose almost completely.

Acetylation of the Polysaccharide—Another lot of polysaccharide (Lot 10), prepared in the manner previously described, contained 2.3 per cent nitrogen. A 0.5 gm. sample of this material was dissolved in 1 cc. of water and then 15 cc. of ethyl alcohol were added. After a few mg. of powdered sodium acetate were added to the cloudy solution and it was allowed to stand for 2 hours, the precipitated polysaccharide was filtered with suction on a Buchner funnel. The slightly moist precipitate was treated with 15 cc. of pyridine and allowed to stand for 6 days with occasional shaking. (Prolonged treatment with pyridine is necessary to swell the particles of the polysaccharide; otherwise the acetylation proceeds with difficulty.) The polysaccharide was then acetylated by gradual addition of 12 cc. of acetic anhydride. The mixture was stirred overnight at room temperature and then for 12 additional hours at 60°. The solution was then poured into a large excess of cold water, whereupon a white precipitate separated out. The acetylated product was filtered and washed with water until free of acid, and dried *in vacuo* at 80°. A yield of 0.33 gm. of the acetylated product was obtained. The acetate was soluble in chloroform, its acetyl value ($COCH_3$) 41.0 per cent, and the nitrogen content 0.92 per cent.

When another lot (No. 11) of the polysaccharide, containing 1.73 per cent nitrogen, was acetylated, a product was obtained with a nitrogen content of 0.95 per cent. The fact that preparations having various nitrogen contents produced acetylated products with a constant nitrogen value indicates that part of the nitrogen exists in organic combination with the polysaccharide.

The specific viscosity, at 23°, of a 0.4 per cent solution of the acetylated polysaccharide in *m*-cresol, was 0.094. By Staudinger's formula with $Km = 10^{-3}$ (13), a molecular weight of approximately 6760 was obtained for the acetylated derivative.

Regeneration of the Polysaccharide from Its Acetate—A sample of 0.15 gm. of the acetate (obtained from Lot 10) was deacetylated with 5 cc. of 50 per cent aqueous methyl alcoholic 3 per cent potassium hydroxide, according to the method previously described (14). The deacetylated polysaccharide was soluble in water and had a nitrogen content of 1.12 per cent. The persistence of the nitrogen after acetylation and deacetylation of the polysaccharide presents additional evidence that part of the nitrogen is not an impurity but probably exists in organic combination with the carbohydrate.

Immunological Properties of the Polysaccharide

Skin Reaction—The culture filtrate gave reactions in sensitive individuals in dilutions up to about 1:10,000. The polysaccharide prepared by fractional precipitation of the free acid of the polysaccharide from alcohol gave positive skin reactions when 0.00001 mg. of the substance was injected intracutaneously. The polysaccharide obtained by regeneration of the acetate gave no reaction when injected in amounts as high as 0.01 mg.

TABLE I

Precipitin Tests with Various Polysaccharide Preparations

Serum	Antigen	Dilution of antigen						
		1:20,000	1:40,000	1:80,000	1:160,000	1:320,000	1:640,000	1:1,280,000
A	Culture filtrate	++++	++	+	0	0	0	0
	Alcohol-pptd. polysaccharide	++++	++++	++++	++++	++	+	0
	Regenerated polysaccharide	++++	++++	++++	++++	++	+	0
B	Culture filtrate	++++	+++	+	0	0	0	0
	Alcohol-pptd. polysaccharide	++++	++++	++++	++++	++	+	0
	Regenerated polysaccharide	++++	++++	++++	++++	++	+	0
Pleural fluid	Culture filtrate	++++	++++	++	0	0	0	0
	Alcohol-pptd. polysaccharide	++++	++++	++++	++++	+++	+	0
	Regenerated polysaccharide	++++	++++	++++	++++	+++	+	0
Normal serum	Culture filtrate	0	0	0				
	Alcohol-pptd. polysaccharide	0	0	0				
	Regenerated polysaccharide	0	0	0				

++++ = complete precipitation; + = partial precipitation; 0 = no precipitation.

When unacetylated polysaccharide was treated with alcoholic potassium hydroxide under conditions similar to those used for regenerating the acetate, it also gave no skin reaction.

Complement Fixation—The culture filtrate, but not the polysaccharide prepared by alcohol precipitation, fixed complement in the presence of serum from patients with coccidioidomycosis. The regenerated polysaccharide was not tested.

Precipitative Reaction—The culture filtrate, the alcohol-precipitated polysaccharide, and the regenerated polysaccharide gave positive precipitative reactions. Serum was used undiluted in 0.1 cc. amounts and was mixed with various dilutions of antigen in 0.85 per cent sodium chloride solution buffered at pH 7.0. The tubes were incubated at 37° overnight and observed for a total of 48 hours. Insufficient amounts of sera were available for the optimal dilution method. Data obtained with two sera, and one sample of pleural fluid obtained from patients with coccidioidomycosis, and one sample of normal serum are presented in Table I.

DISCUSSION

The isolation of barium galacturonate from the hydrolysis products of the polysaccharide establishes the presence of galacturonic acid units in this compound. Direct analysis of the polysaccharide yielded 10.5 per cent uronic acid, a value which is in fair agreement with 11 per cent galacturonic acid isolated as the barium salt from the hydrolyzed solution.

The fact that the specific rotation of the hydrolyzed solution was negative after removal of the galacturonic acid, and also that a mixture of glucosazone and an unidentified osazone could be observed, indicates the presence in the polysaccharide of another sugar besides glucose. When the glucose was fermented out from the solution with *Torula monosa*, 30.5 per cent of reducing sugar remained. The galacturonic acid, glucose, and the unidentified sugar therefore exist in the polysaccharide in the approximate ratio of 1:6:3, respectively.

The polysaccharide (Lot 9) contained 3.2 per cent total nitrogen, of which 0.6 per cent could be accounted for as amino acid nitrogen. However, when the polysaccharide was subjected to the specific protein tests (Millon, xanthoproteic, glyoxylic acid, and biuret tests), the results were negative. This suggests that the nitrogenous substance is probably some compound other than protein. Part of the nitrogen in the polysaccharide (Lot 10) persisted throughout the process of acetylation and deacetylation. The average nitrogen content of two different samples of the acetylated polysaccharide was 0.94 per cent, while in the deacetylated product it was 1.12 per cent. When allowance is made for the increase in molecular weight of the polysaccharide due to the acetyl groups introduced, the amount of nitrogen is virtually unchanged. This fact indicates that part of the nitrogen is an integral part of the polysaccharide molecule.

Both the original culture filtrate and the alcohol-precipitated polysaccharide gave positive skin reactions, but the polysaccharide obtained by regeneration from the acetyl derivative and a sample of the alcohol-precipitated polysaccharide treated with alcoholic potassium hydroxide were no longer active. All three preparations gave positive precipitative reac-

tions. These facts allow two interpretations. Since the process of acetylation and regeneration of the polysaccharide resulted in a loss of nitrogen, it is possible that the reduction in nitrogen content was the result of the elimination of the substance responsible for skin reactivity. This explanation would also require that the substance responsible for skin reactivity is different from the substance giving the precipitative reaction. A second possible interpretation is that treatment with alcoholic potassium hydroxide altered some group in the polysaccharide molecule necessary for skin reactivity but not necessarily required for the precipitative reaction. This latter explanation is supported by the loss in skin reactivity of the alcohol-precipitated polysaccharide treated with alcoholic potassium hydroxide. It seems probable that the latter interpretation is correct in view of the fact that it was not possible to detect the presence of protein in any of the preparations.

The loss in ability of the polysaccharide to fix complement after alcohol precipitation may possibly be explained by some alteration of the physical state of the polysaccharide or by a removal of some lipid constituent of the original culture filtrate.

SUMMARY

The preparation and properties of the polysaccharide produced by *Coccidioides immitis* have been described.

The polysaccharide consists of units of galacturonic acid, glucose, and some unidentified sugar. A nitrogenous compound, apparently other than protein, is associated with the polysaccharide.

The polysaccharide gives a positive skin reaction in sensitive individuals. However, the polysaccharide obtained by regeneration from the acetyl derivative no longer produces this skin reaction. Both the original polysaccharide and that obtained from the acetylated derivative give positive precipitative reactions.

The authors wish to thank Dr. C. E. Smith and Miss R. J. Wheatlake of the Department of Public Health and Preventive Medicine, Stanford University School of Medicine, for assistance with some of the skin tests and serological reactions and for many helpful suggestions.

BIBLIOGRAPHY

1. Hirsch, E. F., and D'Andrea, D., *J. Infect. Dis.*, **40**, 634 (1927).
2. Aronson, J. D., Saylor, R. M., and Parr, E., *Arch. Path.*, **34**, 31 (1942). Kessel, J. S., *Am. J. Trop. Med.*, **19**, 199 (1939). Smith, C. E., *Am. J. Pub. Health*, **30**, 600 (1940). Stewart, R. A., and Kimura, F., *J. Infect. Dis.*, **66**, 212 (1940).
3. Seibert, F. B., *J. Biol. Chem.*, **78**, 345 (1928).
4. Van Slyke, D. D., *J. Biol. Chem.*, **12**, 275 (1912); **16**, 121 (1913–14).

5. Hassid, W. Z., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 228 (1937).
6. Browne, C. A., and Zerban, F. W., Physical and chemical methods of sugar analysis, New York, 3rd edition, 718 (1941).
7. Dore, W. H., *J. Am. Chem. Soc.*, **48**, 232 (1926).
8. Link, K. P., and Nedden, R., *J. Biol. Chem.*, **94**, 307 (1931–32).
9. Militzer, W. E., *J. Chem. Education*, **18**, 25 (1941).
10. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).
11. Niemann, C., Schoeffel, E., and Link, K. P., *J. Biol. Chem.*, **101**, 337 (1933).
12. Hassid, W. Z., and McCready, R. M., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 683 (1942).
13. Staudinger, H., Die hochmolekularen organischen Verbindungen, Kautschuk und Cellulose, Berlin, 56 (1932).
14. Hassid, W. Z., and Barker, H. A , *J. Biol. Chem.*, **134**, 163 (1940)

THE EFFECT OF CERTAIN DIETARY INGREDIENTS ON THE KEEPING QUALITY OF BODY FAT*

By RICHARD H. BARNES, W. O. LUNDBERG, H. T. HANSON, AND GEORGE O. BURR

(From the Division of Physiological Chemistry, University of Minnesota, Minneapolis)

(Received for publication, May 21, 1943)

In general there are two basic factors of importance governing the resistance of natural fats to rancidification. These are (1) the composition of the component glycerides and (2) the amount and nature of existing natural antioxidants. In the actual practice of preparing animal fats for consumption, as in the rendering process, several other factors such as the presence of enzymes, contamination with prooxidants, and so on are of great importance. However, in the present study these latter factors will not be directly considered.

As it is known that the composition of body fat can be altered by the type of food ingested, it follows that the keeping quality of this fat would also be altered. Indirectly this has been a frequent observation. Animal fats of low melting points are usually more susceptible to oxidative rancidification than the more solid fats. Lea (1, 2) has given evidence that the inclusion of cod liver oil in the food of swine results in an increased susceptibility of the animal's fat to oxidation. It was assumed that traces of cod liver oil acids had been deposited in the fat stores and that they activated the oxidation of the other fat that was present.

Lea has further proposed that stability of animal fat may be influenced by antioxidants in the diet (2). Some indirect evidence that dietary antioxidants may be deposited in fat stores and thus enhance the keeping quality of body fat is found in the observation that ingestion of large amounts of soy beans, although known to increase the iodine value, does not decrease the keeping time of the body fat (3). Natural antioxidants of the soy bean oil may have been deposited in the fat stores and acted to counteract the effect of the increased unsaturation. In an attempt to study the possible stabilization of body fat by dietary antioxidants, Overman (4) fed ascorbic acid and hydroquinone to rats, but found no definite changes in keeping time, although there was some slight indication that ingestion of ascorbic acid improved the stability.

The present investigation was undertaken in an attempt to provide

* Aided by grants from the Hormel Research Foundation and the National Dairy Council.

Presented before the Division of Agricultural and Food Chemistry of the American Chemical Society at Detroit, April, 1943.

further information concerning the influence of diet on the stability of body fat.

EXPERIMENTAL

The general plan of the following experiments was to feed specially prepared diets to albino rats of a given sex. At the appropriate time the rats were killed by etherization and exsanguination from the large abdominal vessels. All fat from the abdomen except that lying in the mesentery was carefully removed and frozen in a cold room at a temperature of $-15°$. When frozen, the fat was wrapped in several layers of coarsely woven muslin and pounded with a hammer. The fat was then transferred to small filter flasks, placed in a water bath at 60°, and evacuated with a water aspirator for about 1 hour. At the end of this time most of the water had been removed from the fat. The fat was then squeezed through several layers of muslin and filtered twice through a diatomaceous earth (Hyflo Super-Cel, Johns-Manville). In some cases it was not possible to carry out subsequent work immediately on the fat samples and they had to be stored at $-15°$ for 4 to 6 weeks. In early experiments filtration with diatomaceous earth was not carried out and it was found that in these samples storage at low temperature caused a marked decrease in keeping time. Filtration through the diatomaceous earth effectively prevented this deterioration during storage. In any one experiment all fat samples were treated in an identical fashion, so that while variations in the keeping time of fats from animals raised on the same regimen may be different from one experiment to another, comparison of different fats in a given study can be made. The keeping time of the fats was judged by measurement of the peroxide accumulation in 1.5 cc. samples which were kept in small glass vials in an oven regulated to $63° \pm 0.5°$. Peroxide values (milli-equivalents per kilo) were determined by the method described by King, Roschen, and Irwin (5). Iodine values (Wijs) were determined on most of the original fat samples. In two experiments such small quantities of fat were recovered from the rats that keeping time was judged by the rate of oxygen absorption of 0.2 cc. samples of fat kept at 100° in a Warburg apparatus. More details will be given in the description of the individual experiments.

Experiment 1. Effect of Three Common Laboratory Diets on Keeping Time of Body Fat—Three groups of five rats each were kept on the following diets from the time of weaning until they were approximately 150 days old: (1) a purified diet of the following percentage composition, sucrose 45, casein 28, lard 18, salts (modified (6)) 4, yeast 5, 2 drops of cod liver oil and 1 drop of wheat germ oil to each rat twice a week; (2) a commercial food widely used in rat colonies (Purina fox chow); (3) a stock diet which

had been used in the rat colony at this laboratory and which had the following composition, whole ground wheat 66.5, casein 15.0, milk powder 10.0, butter (unsalted) 5.2, NaCl 0.8, $CaCO_3$ 1.5, cod liver oil 1.0.

Peroxide accumulation as shown in Fig. 1 indicates a prolonged keeping time of the fat from rats fed the laboratory stock diet. A sharp drop in iodine value of the fat from this group offers one explanation for the increased stability. However, from this experiment alone the possibility of an antioxidant deposition in the fat cannot be ruled out, but regardless of its interpretation this experiment does show that diet can have a rather marked effect on the keeping time of the animal's fat.

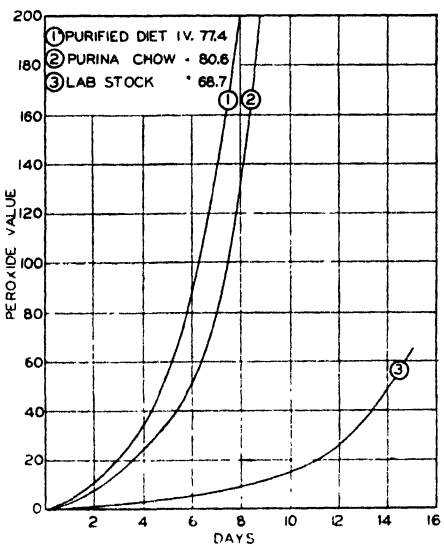


FIG. 1

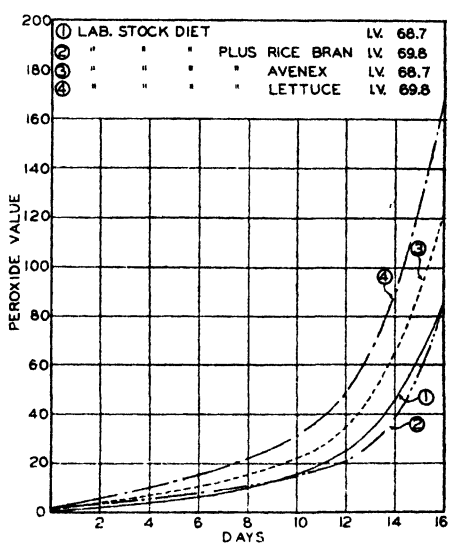


FIG. 2

FIG. 1. The effect of three common laboratory diets on the keeping time of body fat.

FIG. 2. The effect of incorporating several antioxidant sources in the diet.

Experiment 2. Effect of Incorporating Several Antioxidant Sources in Diet—Four groups of five rats each which had been raised on the laboratory stock diet from the time of weaning until they were approximately 150 days old were fed for 30 days as follows: Group 1 was continued on the stock diet and served as controls, Group 2 received 10 per cent rice bran, Group 3, 10 per cent avenex (an oat product), and Group 4, fresh lettuce *ad libitum*. Rice bran has been recently shown to have some antioxidant properties (7); avenex has been claimed to act as an antioxidant for several fats (8); and lettuce has been known for some time to be a source of inhibitols (9). As indicated in Fig. 2, supplementation of the diet with these antioxidant substances did not increase the keeping time of the body fat.

Experiment 3. Influence of Protein Level and Presence of Yeast in Diet—The purified diet described in Experiment 1 was used as a basic diet for this study. By isocalorically exchanging casein and glucose and also providing for a 50 per cent protein content of yeast, the high protein diets were made to contain 30 per cent protein and the low protein diets 15 per cent protein. In one low and one high protein diet the 5 per cent yeast was replaced by synthetic vitamins, so that in the absence of yeast the diet still contained the same absolute amounts of those B vitamins which are known to be necessary for the rat. These four diets were fed from the time of weaning until the rats were approximately 150 days old. From Fig. 3 it is seen that none of these alterations in diet influenced the keeping time of the body fat. The high protein diets gave more rapid growth, but the amount of fat deposited was about the same for each group. Yeast has been recently shown to have antioxidant properties (7, 10) but it is seen here that its presence in the diet is not necessary for the normal stability of body fat.

Experiment 4. Influence of Different Types of Dietary Fat—As a basic diet for this study the purified diet described in Experiment 1 was again used. The fat was altered so that Group 1 received lard (Hormel); Group 2, corn oil (Mazola); Group 3, hydrogenated vegetable oil (Crisco); and Group 4, fresh butter fat. As seen in Fig. 4, the rats receiving butter fat had the most stable body fat. At the same time the iodine value of this fat is the lowest of the four groups. This indication of an altered composition of the body fat offers a possible explanation for the prolonged keeping time of the body fat in this group. As the laboratory stock diet also contained butter fat and provided for a stable fat, it is probable that the butter fat content of this diet was the factor responsible for the increased keeping time noted in Experiment 1. The ingestion of Mazola and Crisco provided a better body fat than lard. As the iodine value of the fat from rats receiving both of these fats was higher than that of the lard-fed animals, it is probable that antioxidants, naturally occurring in these two fats, had been deposited in the fat depots. Some such factor must have been acting to counteract the influence of the increased unsaturation.

Experiment 5. Influence of Tocopherols, Wheat Germ Oil, and Hydroquinone—Four groups of rats which had been raised on a commercial diet (Purina fox chow) from the time of weaning until they were approximately 150 days old were used in this experiment. They were transferred to the purified diet described in Experiment 1, which contained 18 per cent lard (Hormel). Group 1 was continued on this diet without supplementation for 30 days; Group 2 received 0.25 cc. of 15 per cent mixed tocopherols (Distillation Products, Inc.) twice weekly; Group 3 received 0.5 cc. of wheat germ oil (viobin) twice weekly; and Group 4 had 50 mg. of hydro-

quinone mixed in 100 gm. of diet, providing about 5 mg. of the compound each day. Supplementation of diets with tocopherols and wheat germ oil has been carried out in two additional experiments that are not described here. In both of these experiments the results were comparable to those shown in Fig. 5. The slight stabilizing effect of the tocopherols noted in Fig. 5 was not a regular finding. In confirmation of the work of Overman, hydroquinone ingestion did not increase the stability of the body fat.

Experiment 6. Influence of Rancid Fat in Diet from Time of Weaning—Two groups of rats were raised on the high protein-synthetic vitamin B diet described in Experiment 4, from the time of weaning until they were

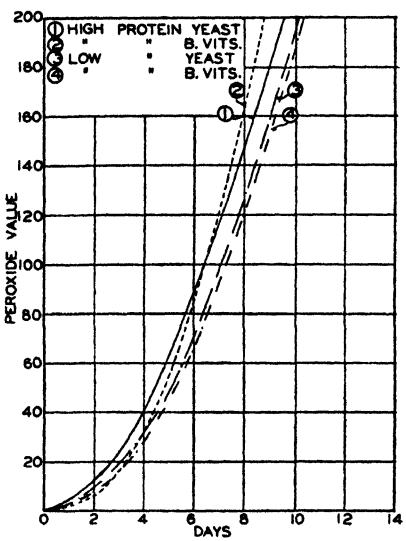


FIG. 3

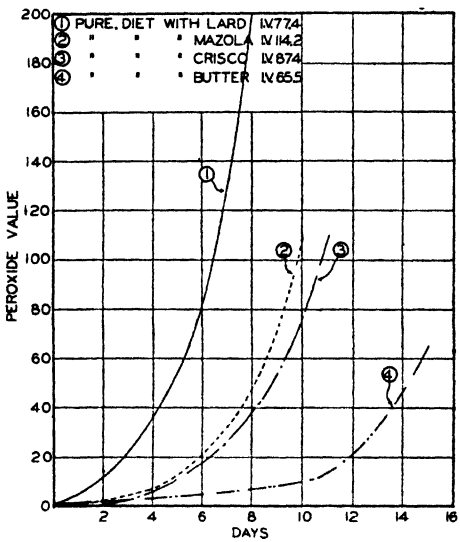


FIG. 4

FIG. 3. The influence of the protein level and the presence of yeast in the diet.
FIG. 4. The influence of different types of dietary fat.

approximately 80 days of age. In Group 1 the lard was protected from any deterioration by refrigeration and in Group 2 the lard was heated and aerated until it had reached a peroxide value of 200 to 400. As seen in Fig. 6, the body fat of the rats receiving rancid fat had a decreased keeping time. The effect of the rancid lard in the diet might have been brought about by the absorption and deposition of certain prooxidants in the fat tissues or through the destruction of dietary antioxidants that are essential for the natural stabilization of body fat.

Experiment 7. Influence of Ingesting Rancid Fat by Adult Rats—Three groups of rats were raised on a commercial food (Purina fox chow) until they were approximately 150 days old. The rats were then fasted for 1

week and given the purified diet described in Experiment 1. At this time Group 1 was killed and Groups 2 and 3 were given the same diet, but the lard had been rancidified to a peroxide value of about 200. Group 2 was killed after 2 weeks and Group 3 after 4 weeks on the rancid lard diets. In this case there was no reduction in the keeping time of the body fat as a result of ingestion of rancid lard (Fig. 7). This indicates that the reduction of keeping time noted in Experiment 6 was not due to the absorption and deposition of prooxidants, for if such had been the case the body fat in the present experiment would also have shown a reduced keeping time. The alternative explanation that the rancid fat destroyed necessary anti-

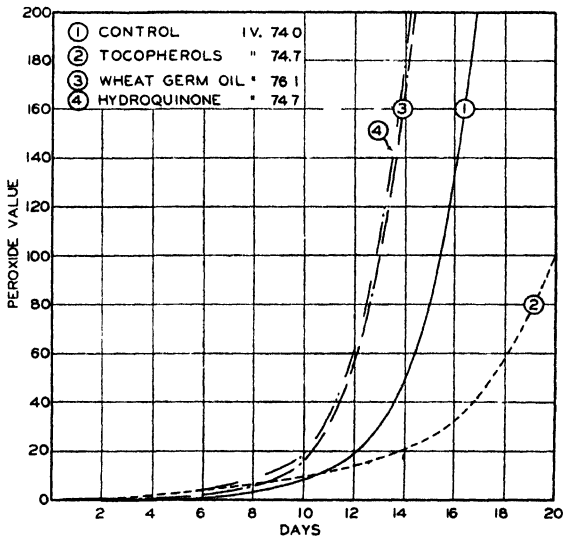


FIG. 5. The influence of tocopherols, wheat germ oil, and hydroquinone

oxidants which were in the diet leads to two postulations. First, the natural stability of body fat necessitates the presence of antioxidants in the diet, and second, naturally occurring antioxidants in the body fat are stored and do not require constant replenishment. Proof of the first postulation is offered in the succeeding two experiments.

Experiment 8. Influence of Vitamin E-Deficient Diet—A group of rats was raised from the time of weaning until they were approximately 100 days old on a diet which was free of vitamin E and fat. The percentage composition of the diet was sucrose 84, casein 12, and salts (11) 4. One-fourth teaspoonful of yeast into which carotene and viosterol were mixed was supplied each day. For 2 weeks before the termination of the experiment each animal received a daily dose of 10 drops of the ethyl esters of

corn oil fatty acids completely free from unsaponifiable matter. This was given in order to stimulate fat synthesis, because the fat-free diet had almost completely inhibited fat deposition. For controls a group of rats which had been raised on the commercial food was used. The amount of fat in the vitamin E-deficient rats was so small that the previously used technique of measuring peroxide accumulation could not be used. Instead, a standard Warburg apparatus with glycerol in place of water as a heating bath and with light mineral oil in place of the usual manometer fluid was used. The regular Warburg respiration flasks containing 0.2 cc. of the test fat were connected to the manometers with mineral oil as a lubricant

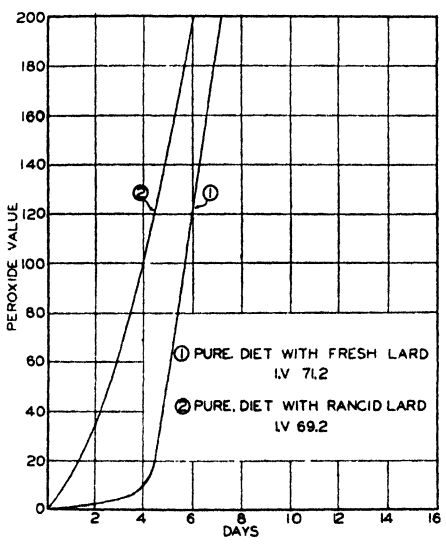


FIG. 6

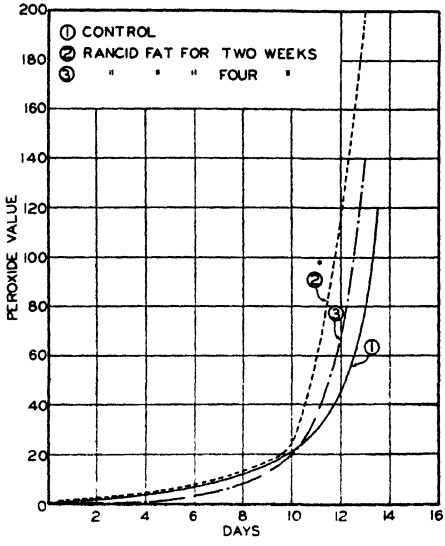


FIG. 7

FIG. 6. The influence of rancid fat in the diet from the time of weaning.
FIG. 7. The influence of ingesting rancid fat by adult rats.

and then placed in the glycerol bath at 100°. The system was filled with tank oxygen and the manometers set in motion. Readings and corrections were applied as in normal studies on respiration. In Fig. 8 the oxygen absorption values are given in arbitrary units, but the relative keeping times are clearly indicated. The reduced keeping time of the body fat from the vitamin E-deficient animals gives added proof that the natural stability of the rat's fat is dependent upon antioxidants derived from the diet. Furthermore, this experiment indicates the important rôle that vitamin E may play in this stabilization.

Experiment 9. Effect of α-Tocopherol and Hydroquinone on Stability of Fat in Vitamin E-Deficient Rats—Since the ingestion of a vitamin E-free

diet sharply reduced the keeping time of body fat and as in previous studies it had been found that the administration of supplemental tocopherols did

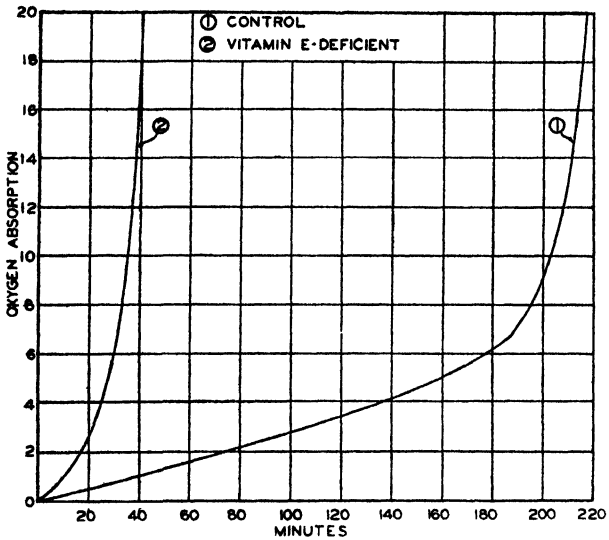


FIG. 8. The influence of a vitamin E-deficient diet

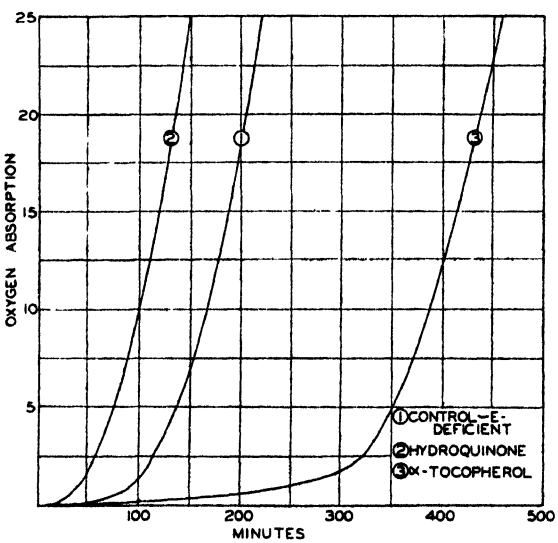


FIG. 9. The effect of α-tocopherol and hydroquinone on the stability of fat in vitamin E-deficient rats.

not extend the keeping time of normal fat, the following experiment was carried out. Three groups of rats that had been raised from the time of

weaning until they were approximately 80 days old on a diet which was free of both fats and vitamin E were given daily supplements of the ethyl esters of corn oil fatty acids for 30 days. The rats in Group 1 were then given by stomach tube 2 cc. of the methyl esters of corn oil acids. Group 2 in a like manner received 50 mg. of hydroquinone dissolved in water and 2 cc. of the corn oil esters. Group 3 received 200 mg. of α-tocopherol dissolved in 2 cc. of the corn oil esters. The dose of hydroquinone proved lethal for one rat in Group 2 and two others exhibited signs of some toxicity for 1 day following its administration. 6 days after the supplements were given, the rats were killed and their abdominal fat prepared for measurements of oxygen absorption. In this experiment a slightly different flask from that used in Experiment 8 was employed and the test was carried out with the flasks filled with air rather than oxygen. For these reasons keeping times in Experiments 8 and 9 are not comparable. The very short induction period for the vitamin E-deficient rats is again seen (Fig. 9). Hydroquinone did not increase the keeping time, but appeared to exert a deleterious effect. α-Tocopherol markedly increased the keeping time, but fat from rats raised on commercial food and studied under conditions which were comparable to those employed in this experiment have indicated that the α-tocopherol had not brought about a return of the keeping time to normal.

DISCUSSION

These experiments have demonstrated the very wide variations in keeping time of body fat that may be brought about by changes in diet. One pure compound, α-tocopherol, has been found to play an important rôle in the stabilization of animal fats. However, there appears to be some peculiarity in the over-all influence of this substance in the diet. If the diet is completely free of tocopherols and other possible antioxidants that occur in fats, the stability of the body fat is sharply reduced. In fact it is likely that in this fat antioxidant protection is completely absent. If α-tocopherol is supplied to these animals in one enormous dose, the keeping time is increased, indicating that dietary tocopherols are actually deposited in the fat stores and offer protection against oxidation. However, the keeping time of fat from rats receiving a normal vitamin E-containing diet is not increased by feeding supplemental vitamin E, either as tocopherols or wheat germ oil. As the keeping time of a carefully rendered lard may be substantially increased by the direct addition of small amounts of either mixed tocopherols or wheat germ oil, it is not clear why the keeping time of body fat should not be enhanced by ingesting larger quantities of the tocopherols. Another factor that makes the interpretation of the influence of dietary antioxidants obscure is the observation that ingestion of corn oil increases the keeping time of the body fat even though the iodine

value of this fat is increased almost 2-fold. The mechanism of stabilization of body fat by dietary antioxidants is certainly not clear, but one conclusion does seem to be justified. The naturally occurring antioxidants in body fat of the rat cannot be synthesized by the animal, and, therefore, like the vitamins, must be supplied by the diet.

SUMMARY

1. The natural stability of body fat from rats receiving a normal diet can be increased but this increase has been found to be relatively small and it is probably due to changes in both glyceride composition and antioxidant content brought about by alterations in the diet.

2. Dietary supplements of lettuce, avenex, rice bran, yeast, casein, hydroquinone, mixed tocopherols, or wheat germ oil do not increase the keeping time of body fat which already possesses a normal stability.

3. The keeping time of body fat of the rat is markedly reduced by the continued ingestion of a diet which is free of vitamin E and other sources of fat-soluble antioxidants. It is proposed that, in the rat, antioxidants of the body fat are derived solely from the diet.

4. The ingestion of certain antioxidant substances such as yeast and hydroquinone does not restore the normal stability to body fat from vitamin E-deficient rats, but α-tocopherol effects such a restoration.

5. Prooxidants of rancid fat are not stored in the fat depots, but if such fat is ingested throughout the growing period of a rat, body fat stability is reduced, presumably owing to destruction of the dietary antioxidants.

6. Naturally occurring antioxidants in the fat depots do not require frequent replenishment from the diet, but are stored for relatively long periods.

BIBLIOGRAPHY

1. Lea, C. H., *J. Soc. Chem. Ind.*, **50**, 343 (1931).
2. Lea, C. H., Rancidity in edible fats, New York, 27 (1939).
3. Dyme, H. C., Nelson, P. M., Lowe, B., and Nelson, B. E., *Iowa State Coll. J. Sc.*, **15**, 189 (1941).
4. Overman, A., *J. Biol. Chem.*, **142**, 441 (1942).
5. King, A. E., Roschen, H. L., and Irwin, W. H., *Oil and Soap*, **10**, 105 (1933).
6. Hubbel, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, **14**, 273 (1937).
7. György, P., and Tomarelli, R., *J. Biol. Chem.*, **147**, 515 (1943).
8. Olcott, H. S., *Oil and Soap*, **18**, 77 (1941).
9. Bradway, E. M., and Mattill, H. A., *J. Am. Chem. Soc.*, **56**, 2405 (1934).
10. Clausen, D. F., Barnes, R. H., and Burr, G. O., *Proc. Soc. Exp. Biol. and Med.*, in press.
11. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

ADDITIONAL OBSERVATIONS ON THE CHICK ANTIANEMIA VITAMIN*

By BOYD L. O'DELL and ALBERT G. HOGAN

(From the Department of Agricultural Chemistry, University of Missouri, Columbia)

(Received for publication, May 20, 1943)

While attempting to devise a simplified ration that is adequate for the chick, Hogan and Parrott (1, 2) observed that on certain rations the number of red blood cells was distinctly subnormal. The anemia was of the hyperchromic, macrocytic type, and it was due to the lack of an unrecognized vitamin which was designated vitamin B_c. An aqueous extract of liver is one of the best sources of the vitamin. It is adsorbed from acid solution on fullers' earth and is easily destroyed by heat in acid solution. The essential features of the above report were confirmed by Mills, Briggs, Elvehjem, and Hart (3) who also have shown that chicks maintained on a simplified diet require an unrecognized factor to promote growth, hemoglobin formation, and normal feathering.

The purpose of the present investigation was (1) to improve the diet used for producing anemic chicks, (2) to develop a technique for the assay of vitamin B_c, and (3) to obtain additional information on its chemical properties.

EXPERIMENTAL

Day-old white Leghorn chicks, obtained from the University poultry department, were divided into groups of eight and placed in electrically heated batteries. Water and the experimental ration were supplied *ad libitum*. The chicks were weighed at regular intervals. When they were 3 weeks of age, and each week thereafter, blood samples were taken to determine the degree of anemia. Very few chicks were anemic at 2 weeks of age. The chicks which had not become anemic by the end of the 5th week were discarded and the red cell volume was recorded as normal.

Biological Studies

Criteria of Anemia—Hogan and Parrott (2) used erythrocyte counts and hemoglobin content to determine the degree of anemia, but counting erythrocytes is quite laborious and there was considerable variability in reading the visual colorimeter used at the time in making hemoglobin determina-

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series, No. 894. Aided by a grant from Parke, Davis and Company.

tions. In an effort to eliminate these objectionable features, determination of the red cell volume by use of the Van Allen hematocrit tube (4) was found to be quite rapid and efficient. In this determination a measured volume of blood is mixed in a Van Allen tube with a 1.3 per cent solution of sodium oxalate and centrifuged until the erythrocytes are solidly packed. This is accomplished by centrifuging for 20 minutes in an International centrifuge, size 1, at a speed of 2500 R.P.M.

In order to determine the relation between red cell count, hemoglobin content, and the hematocrit value, these three determinations were made on over 60 samples of blood taken at random from chicks on anemia-producing rations. The results by all of the methods showed quite close correlation. An attempt to derive a mathematical relation between the red cell volume in per cent and the red cell count, according to the method of Mills (5), gave the equation, $C = 8.42 + 10.42E$, in which C is the red cell volume in per cent and E is the erythrocyte count in millions per c.mm. The coefficient of correlation is 0.85. A similar relationship between the red cell volume and the hemoglobin content is expressed by the equation, $H = 0.66 + 0.18C$, in which H is the hemoglobin content in gm. per 100 cc. of blood and C is the red cell volume in per cent. The coefficient of correlation is 0.94, showing that approximately the same degree of anemia is indicated by these two methods.

The relation between the cell volume and the erythrocyte count, and between the cell volume and hemoglobin content, is shown in Figs. 1 and 2.

Fig. 1 shows that as the anemia develops the rate of decrease in cell volume is accelerated. This indicates that the cells become larger when the anemia begins to develop, though there is no reason to suppose that the cells continue to increase in size as the anemia progresses. Presumably the same type of curve would be obtained if the large cells disappeared more rapidly than those that are smaller. Fig. 2 indicates that as the hemoglobin content decreases the cell volume decreases at a constant rate.

During the first part of the investigation a chick was considered anemic when the hematocrit value was 25 per cent or less. It was found that assays for the curative agent were more reliable if the cell volume was allowed to fall still lower; therefore in the latter part of the study a chick was not classed as anemic until the red cell volume reached the arbitrary value of 20 per cent by volume. This corresponds to a hemoglobin value of 4.26 gm. per 100 cc. of blood and to an erythrocyte count of 1.11 millions per c.mm. The normal hematocrit value for thirty-five chicks 3 to 5 weeks of age, maintained on a practical diet of natural foodstuffs, was 32 per cent. Calculated by the formulae given above this would correspond to an erythrocyte count of 2.26 millions per c.mm. and a hemoglobin content of 6.42 gm. per 100 cc.

Assay Technique—When the red cell volume fell to 20 per cent, or less, the chicks were used to assay experimental materials. They were continued on the basal diet, and the preparation to be assayed was administered orally every other day for a 12 day period. The final hematocrit reading was taken on the 14th day. Dry preparations were administered in gelatin capsules and liquids by pipette. Both positive and negative controls were maintained on the basal diet during the assays. The chick unit of vitamin B_c is defined as the amount that must be administered every

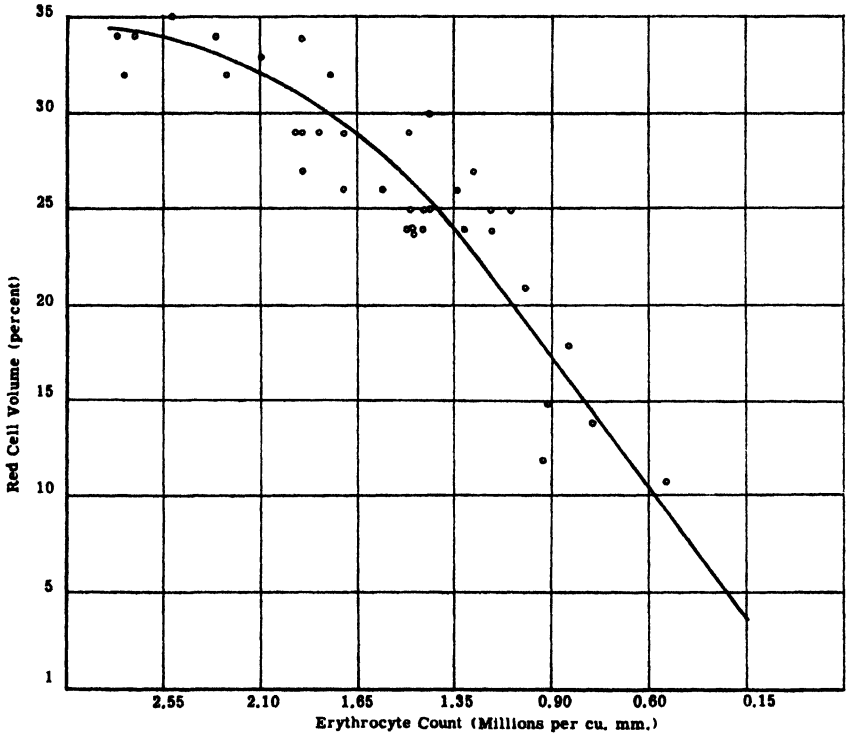


FIG. 1. Red blood cell volume is not a linear function of the red cell count

other day for six doses to bring the hematocrit reading from 20 per cent or below to 30 per cent or above in at least 60 per cent of the chicks. Chicks which did not survive the first 5 days of the assay period were disregarded, but those which died after the 5th day were classified as failures.

Rations Used for Production of Anemia—The diets were of the simplified type, consisting of casein, corn-starch, salts, lard, vitamins, and a liver extract fraction. The diet of Hogan and Parrott contained tikitiki and a 95 per cent alcohol extract (Liver Extract 3703) of dried pork liver as sources

of the B vitamins other than thiamine and riboflavin. As other synthetic vitamins became available, it was found that more uniform results were obtained when tikitiki was omitted and pyridoxine was included. However, the alcoholic liver extract could not be dispensed with until quite recently, when it was found that it could be replaced by biotin and other known members of the vitamin B complex. As previously mentioned, during the first part of this investigation the chicks were considered anemic when a hematocrit value of 25 per cent by volume was reached instead of

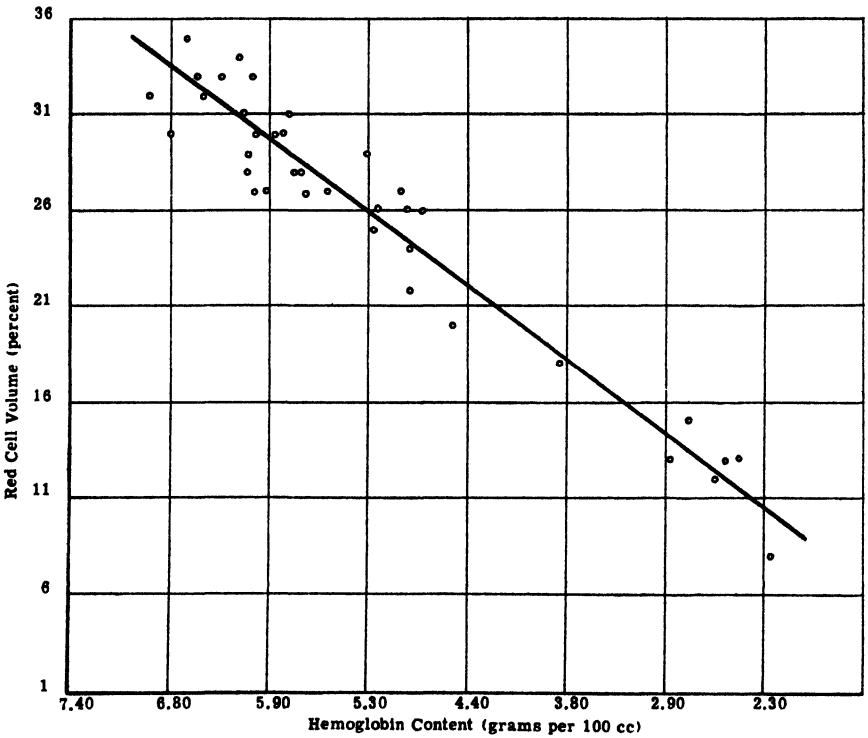


FIG. 2. Red blood cell volume is a linear function of the hemoglobin content

the present standard of 20 per cent. The data will therefore be presented in two sections, corresponding to the standard by which the chicks were classified as anemic.

1. A red cell volume of 25 per cent was the standard. Table I gives the composition of diets employed during this part of the study. Tikitiki was first replaced by pyridoxine in Ration 6636. This diet contained only 100 γ of pyridoxine in addition to that supplied by Liver Extract 3703. Optimum growth is not supported by this amount, but large amounts

of the vitamin apparently decrease the incidence of anemia. This is indicated by a comparison of the response of chicks from a flock restricted to dry feed which received Rations 6636 and 6637, shown in Table II.

TABLE I

Composition of Rations

Constituents	Ration 6636	Ration 5056
	per cent	*per cent*
Casein	35	35
Corn-starch	43	45
Salts*	4	4
Cellu flour	3	3
Lard	7	7
Cod liver oil	2	2
Wheat germ oil	4	4
Liver extract 3703	2	
Vitamins added per 100 gm. ration		
	mg.	*mg.*
Thiamine hydrochloride	0.2	0.2
Riboflavin	0.4	0.4
Pyridoxine hydrochloride	0.1	0.3
Calcium pantothenate		1.0
Choline chloride		100.0

* A modification of Salt Mixture 351, described by Hubbell, Mendel, and Wakeman (6). 2.5 gm. of manganous sulfate, $MnSO_4 \cdot 4H_2O$, were added to 100 gm. of Salt Mixture 351.

TABLE II

Incidence of Anemia As Affected by Diet of Laying Flock and of Experimental Chicks

Ration No.	Source of chicks	No. of chicks*	Average weight, 3rd wk.	Mortality	Anemic†
			gm.	*per cent*	*per cent*
6636	Winter flock allowed open range	348	74	27	62
6636	Summer " " " "	70	87	34	40
6636	" " restricted to dry feed	74	73	20	70
6637	" " " " " "	16	90	6	31
5056	Winter " allowed open range	67	70	45	45

* Number surviving at the end of the 1st week.

† A red cell volume of 25 per cent or less at the end of the 5th week.

Ration 6637 was the same as Ration 6636 except that it contained 300 γ of pyridoxine. Approximately 70 per cent of the chicks from the summer flock maintained on Ration 6636 became anemic, while only 31 per cent became anemic on Ration 6637. The higher level of pyridoxine markedly decreased the mortality rate.

Ration 6636 was first employed in the winter months, and during this time about 62 per cent of the chicks became anemic. However, as the spring and summer months came on, the incidence of anemia decreased, dropping to 40 per cent of the total, as is shown in Table II. Although the hens from which the eggs were obtained were allowed free range throughout the year, they would not be able to obtain as much green feed during the winter as during the summer. It was suspected, therefore, that the anti-anemia factor was being stored in the egg from the green feed which the hens consumed during the summer months. To test this hypothesis part of the summer flock was kept indoors and fed a practical laying ration of natural feeds. Ration 6636 was fed to chicks from this flock and the incidence of anemia increased to 70 per cent, which is slightly better than was obtained during the winter months with the flock on open range. Thus it appeared that the factor is abundant in green feeds and is stored in the egg. Thereafter the chicks were obtained from hens restricted to dry feed.

As more synthetic vitamins became available, an effort was made to dispense with Liver Extract 3703. Ration 5056, containing thiamine, riboflavin, pyridoxine, pantothenic acid, and choline, but no liver extract, is an example of such an attempt. The rate of growth was about the same as on the other type of diet, but the mortality rate was excessive. As will be shown later, this was probably due at least partially to a biotin deficiency.

2. A red cell volume of 20 per cent was the standard. It is unnecessary to point out that the use of this standard decreased to some extent the percentage of chicks which were classed as anemic. The composition of the rations used during this phase of the investigation is shown in Table III.

In an effort to produce a larger number of chicks that were useful for assay, Ration 6636 was modified in various ways. The level of pyridoxine was increased to 0.3 mg. per 100 gm. of diet, though the change decreases slightly the incidence of anemia. However, the mortality rate is reduced, and since the survivors are superior test animals the over-all effect is advantageous. Furthermore, the wheat germ oil and cod liver oil were replaced by 2-methyl-1,4-naphthoquinone, α-tocopherol, and a vitamin concentrate which contained vitamins A and D. The effect on the incidence of anemia due to the addition of sulfaguanidine to the diet was also studied. Our observations are summarized in Table IV.

It will be noted that in addition to the column showing the percentage of anemic chicks, a column is included which indicates the percentage of chicks which were usable for assay purposes. A useful chick is defined as one which has a red cell volume of 20 per cent or less and survives the first 5 days of the period during which supplements are administered. This information is helpful in evaluating a basal ration, since a diet may produce

TABLE III

Composition of Rations

Constituents	Ration No.			
	6091	6385	6554	6582
	per cent	*per cent*	*per cent*	*per cent*
Casein	35	35	25	25
Gelatin			10	10
Corn-starch	43	43		
Dextrin			56	56
Cellu flour	3	3		
Lard	12.5	12.5		
Soy bean oil			4.5	
Wheat germ oil				4.5
Salts*	4	4	4	4
Liver Extract 3703	2	2		
Mixture 6092 (vitamins A, D, E, K)†	0.5	0.5	0.5	0.5
Vitamins added per 100 gm. ration				
	mg.	*mg.*	*mg.*	*mg.*
Thiamine hydrochloride	0.4	0.4	0.4	0.4
Riboflavin	0.8	0.8	0.8	0.8
Pyridoxine hydrochloride	0.3	0.3	0.3	0.3
Calcium pantothenate	2	2	2	2
Choline chloride	200	200	200	200
Nicotinic acid			10	10
Inositol			100	100
			γ	*γ*
Biotin (S. M. A. No. 200)			10	10

* See foot-note to Table I.

† 0.5 per cent of Mixture 6092 furnished 7250 I.U. of vitamin A, 725 I.U. of vitamin D, 0.1 mg. of 2 methyl-1,4-naphthoquinone, and 8 mg. of α-tocopherol. Natola, supplied by Parke, Davis and Company, was dissolved in corn oil and served as the source of vitamins A and D, while vitamins E and K were added as synthetic compounds.

TABLE IV

Incidence of Anemia As Affected by Diet of Experimental Chicks

Ration No	Sulfaguanidine added	No. of chicks*	Average weight, 3rd wk.	Mortality	Anemic†	Usable for assay
	per cent		*gm.*	*per cent*	*per cent*	*per cent*
6091	0	206	95	21	23	21
6091	1	92	68	23	60	39
6385	0	88	81	13	24	14
6385	0.5	137	67	28	37	21
6385	0.25	85	73	10	53	46
6554		57	92	15	51	51

* Number surviving at the end of the 1st week.

† A red cell volume of 20 per cent or less at the end of the 5th week.

a high proportion of anemic chicks but be impractical because of a high mortality rate during the assay period.

It should be stated that there is considerable variation in the incidence of anemia. This is probably due to at least two factors: (1) the chicks themselves, and (2) the alcoholic extract of liver, No. 3703. That the source of the chicks affects the incidence of anemia has already been shown. Even when the diet of the hen is controlled, there is variation in the chicks from one hatch to another. Although the liver extract is prepared in as nearly the same manner as possible each time, the chicks do not respond to different preparations in exactly the same way. Ration 6091, which contains 0.3 mg. of pyridoxine per 100 gm. of diet and the vitamin A, D, E, and K mixture instead of the oil carriers, produced a rather low incidence of anemia, 25 per cent. However, it should be mentioned that although relatively few chicks were anemic according to the arbitrary standard many of the others had low hematocrit values and might be classed as anemic under less severe standards. Ration 6385 is similar to Ration 6091 except that it does not contain choline. The incidence of anemia on this ration was about the same as on Ration 6091, but the percentage of usable chicks was somewhat lower.

The inclusion of sulfaguanidine in some of the rations was based on the theory that bacterial synthesis in the alimentary tract might make the antianemia factor available to the chick and thus reduce the incidence of anemia. Since Black, McKibbin, and Elvehjem (7) have used sulfaguanidine successfully to prevent bacterial synthesis in the intestine of the rat, this drug was added to the chick rations at the levels indicated in Table IV. When it was added to Ration 6091 at a level of 1 per cent, the incidence of anemia was increased from 23 to 60 per cent without an increase in mortality. There was a decrease in weight, presumably because of a reduction in the number or amount of vitamins that were available to the chick. The chicks would still respond to treatment with the vitamin B_c concentrates, which indicates that it was not entirely a toxic effect. The addition of 0.25 per cent sulfaguanidine to Ration 6385 increased the anemia from 24 to 53 per cent. The addition of 0.5 per cent did not prove as useful as 0.25 per cent because of a higher mortality rate.

Almquist, Stokstad, Mecchi, and Manning (8) and Hegsted *et al.* (9) have shown that chicks require more glycine and arginine than is supplied by casein. Since our basal diet appeared to be deficient in these amino acids, 10 per cent gelatin was substituted for that amount of casein in Ration 6554. This change improved the growth rate and feathering, and thus produced a better test animal. Another improvement was made by discarding Liver Extract 3703, and adding a biotin concentrate.[1] This modification eliminated one of the variables, the liver extract, and made it

[1] Concentrate No. 200, obtained from the S. M. A. Corporation.

possible to obtain a high incidence of anemia without the use of sulfaguanidine. Furthermore the chicks produced were superior in appearance and the mortality rate was reduced.

When the new type of diet, Ration 6554, containing the biotin concentrate was first tried, soy bean oil was used as a source of fat. Although this diet produced a fairly high proportion of anemic chicks, our past experience indicated that the substitution of wheat germ oil for soy bean oil increased the incidence of anemia. It was decided therefore to study the effect of various sources of fat on the incidence of anemia among chicks under observation at the same time. These data, not included in Table IV, are summarized in Table V.

Evidently chicks may at times become severely anemic on rations that contain soy bean oil, but up to the present the results indicate that the anemia develops much more consistently when the soy bean oil is replaced by wheat germ oil. Ration 6632 is similar to Ration 6582 except that the

TABLE V

Incidence of Anemia As Influenced by Source of Fat in Diet

Ration No.	Source of fat	No of chicks	Average weight, 3rd wk.	Mortality	Anemic	Usable for assay
			gm	*per cent*	*per cent*	*per cent*
6554	Soy bean oil, 4.5%	47	97	21	38	30
6582	Wheat germ oil, 4.5%	46	87	15	65	59
6632	Lard, 4.5%	15	67	33	66	20

wheat germ oil is replaced by lard. When lard was used as a source of fat, all of the surviving chicks became anemic, but the growth rate was low and the mortality rate was quite high. Since many of the chicks which became anemic died within a week, the percentage usable for assay was very low. It appears that the oils contain a factor essential for chicks maintained on this type of diet and that it is more abundant in soy bean oil than in wheat germ oil. It may not be identical with vitamin B_c.

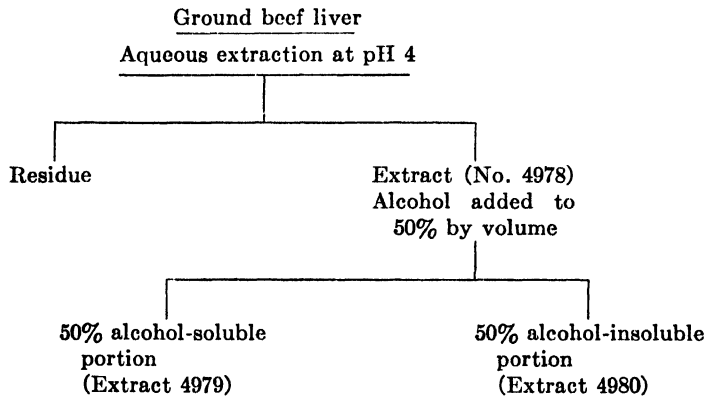
Although the chicks develop anemia more readily when the liver extract is omitted, it is also true that they respond to treatment less readily. This difference may be due to the vitamin B_c content of the liver extract, or to some other limiting factor which is supplied by this extract.

Chemical Studies

Fractionation Procedures[2].—An aqueous extract of beef liver seemed to be the most potent source of the vitamin. In most of the fractionation studies

[2] A preliminary announcement of the isolation of crystalline vitamin B_c was recently reported (10).

an extract prepared according to the following procedure was used. 50 pounds of fresh, ground beef liver were stirred with 15 gallons of boiling water adjusted to pH 4.0. The temperature was maintained at 90° for 5 to 10 minutes; then the solution was allowed to settle. The supernatant liquid was decanted off and the residue filtered. This process was repeated with 10 gallons of water. The combined extracts (No. 4978) were concentrated *in vacuo* to approximately 4 liters, and sufficient 95 per cent alcohol was added to make a 50 per cent solution. The precipitate from this treatment was washed three to four times with 50 per cent alcohol and the washings were added to the original filtrate. The 50 per cent alcohol-soluble portion (Extract 4979) was concentrated to a syrup *in vacuo*, and constituted the source material for further fractionation. A curative unit



of Extract 4979 was 400 mg. In more recent work an aqueous extract of pork liver corresponding to Extract 4978[3] has been used.

The procedure for the preparation of a concentrate of vitamin B_c is as follows: 1 kilo of Extract 4979 is dissolved in 10 liters of water and the solution is adjusted to pH 1 with dilute sulfuric acid. Any insoluble residue is removed by decantation and centrifuging. 200 gm. of English fullers' earth are added to the filtrate and stirred for 1 hour. The earth is removed, washed three times with water, and the process is repeated with another 200 gm. of fullers' earth. The combined adsorbates are then eluted with 0.2 N ammonium hydroxide, three portions of 3 liters each being used. The combined eluates are concentrated to a syrup under reduced pressure. The material contains 1 unit in 40 mg. and represents a yield of about 45 per cent of the activity in the original extract. It is then dissolved in water to make a 5 per cent solution, adjusted to pH 1, and the flocculent material that does not dissolve is removed by centrifuging. The clear

[3] Supplied by Parke, Davis and Company.

filtrate is then adsorbed three times with a total amount of Super Filtrol[4] equivalent to twice the weight of solids. After washing, the filter cake is eluted with 1 per cent ammonia in 50 per cent alcohol, with three portions of 800 cc. each. The eluates are combined and concentrated to a convenient volume. A unit of this material is about 15 mg., corresponding to a recovery of about 80 per cent of the activity of the previous fraction. In some cases precipitation with phosphotungstic acid was introduced at this point. The material is dissolved in water to make a 5 per cent solution and is adjusted to pH 1. A 10 per cent sodium phosphotungstate solution is added to the point of maximum precipitation. The mixture is allowed to stand in the refrigerator overnight, and the precipitate is centrifuged off and washed several times with water. The precipitate is decomposed by grinding in a mortar with a paste of barium hydroxide, and then extracted

TABLE VI

Concentrates Prepared from Liver Extract 4979

Description	Concentration effected	Recovery
		per cent
Eluate from readsorption with Super Filtrol.	20	36
10% ammonia eluate of norit adsorption at pH 3	7	22
10% " " " Amberlite IR4* adsorption at pH 4.5.	4	40
Barium hydroxide ppt.	2	25
Zinc sulfate ppt.	4	36
Phosphotungstic acid ppt.	5	50
Residue from cold methanol extraction	2	60
Methanol ppt. of phenol solution	5	95

* The use of Amberlite IR4 was suggested by Dr. J. J. Pfiffner.

with a hot solution of barium hydroxide. This process is repeated until no more color is removed. The barium is removed from both the filtrate and the decomposed precipitate as barium sulfate. A unit of the phosphotungstic acid precipitate is 7 mg. and approximately 70 per cent of the activity of the previous fraction is recovered. This is about the same degree of concentration as is obtained by a second readsorption on Super Filtrol. The over-all yield is about 25 per cent, and the factor has been concentrated 55 to 60 times.

Other procedures which have effected some concentration of the factor when applied to Extract 4979 are presented in Table VI, but in most cases the recovery was too low to make the procedure useful for the purpose of

[4] The Super Filtrol was obtained from the Filtrol Corporation, 315 West Fifth Street, Los Angeles.

preparing a concentrate. Phosphotungstic acid precipitation offers some promise, but the use of this reagent would not be practical in the precipitation of crude extracts. The precipitation of a phenolic solution of the anti-pernicious anemia factor with a large volume of methanol was found useful by Ungley (11) in preparing concentrates of that factor. This procedure also proved helpful in concentrating vitamin B_c.

Properties of Vitamin B_c. Stability—The factor is much more stable in alkaline than in acid solution. Approximately 50 per cent of the activity is lost by allowing a solution at pH 1 to stand at room temperature for 72 hours. At least 80 per cent of the activity is lost by acid autoclaving for 2 hours, whereas less than 50 per cent is destroyed by alkaline autoclaving. Less than 30 per cent of the factor is destroyed by standing in a 5 per cent solution of hydrogen peroxide at room temperature for 12 hours.

Precipitants—The factor is partially precipitated from a 10 per cent solution of Extract 4979 with salts of lead, mercury, barium, zinc, and silver. Considerable loss is associated with lead and mercury precipitation. However, no loss is sustained in precipitation with zinc, but less than 40 per cent of the factor is precipitated.

Phosphotungstic acid gives almost complete precipitation.

Solubility—The factor as it occurs in crude concentrates is insoluble in the common organic solvents such as ether, chloroform, pyridine, ethanol, butanol, and acetone. It is very slightly soluble in dioxane and quite soluble in glacial acetic acid, phenol, and hot methanol.

Adsorbents—The factor is much more completely adsorbed on fullers' earth from acid than from alkaline solution, but there is considerable loss associated with acid adsorption. Since the factor is quite labile to acid, the loss during adsorption can be at least partially attributed to destruction by hydrogen ions. However, acidification following the preliminary adsorption does not lead to such marked losses. In view of the fact that the factor is destroyed at pH 1, attempts were made to adsorb it on fullers' earth from more alkaline solutions. Less than one-third as much of the activity was recovered by adsorption at pH 3.4 as at pH 1, owing to incomplete adsorption. Practically none is adsorbed at pH 7. Readsorption was carried out effectively with Super Filtrol which gave a 2- to 3-fold concentration and recovered approximately 80 per cent of the activity. The factor is completely eluted from fullers' earth with a dilute ammonia solution.

Norit, Nuchar, and Darco will remove the factor almost completely from solution at pH 5, but the recovery was unsatisfactory. It was not determined whether the loss was due to destruction or to incomplete elution. The factor is eluted with difficulty, 5 to 10 per cent ammonia being required for the most complete elution.

Amberlite IR4, an acid adsorbent described by Myers, Eastes, and Myers (12), adsorbs the vitamin, and it may be eluted with 5 per cent ammonia to give a 40 to 50 per cent recovery. Apparently aluminum oxide adsorbs the factor, but it has not yet been possible to recover it. Permutit and kaolin have not shown any promise as adsorbents.

Comparison of Vitamin B_c with Other Antianemia Factors—Simmons and Norris (13) have reported that xanthopterin will cure a nutritional anemia which develops in Chinook salmon maintained on a high protein diet. Inasmuch as the properties of xanthopterin resembled those of vitamin B_c, it was prepared synthetically according to the method of Purrmann (14). It showed no curative action on the chick anemia.

A sample of a potent fraction[5] containing the anti-pernicious anemia factor showed no activity for the chick.

DISCUSSION

This investigation confirms the conclusions reported by Hogan and Parrott (2), but there is one discrepancy that may require some comment. The incidence of anemia they observed was higher than that given in this report. In explanation it may be stated that (1) the percentage of anemic chicks reported by Hogan and Parrott was based on the number of chicks that survived, while in this paper it is based on the total number started; (2) in order to have chicks that are more suitable for assay purposes the standard for anemia was made more severe; (3) all of the observations of Hogan and Parrott were made in the winter and early spring, when presumably the laying flock did not have access to green feed; the data reported in this paper were collected throughout the year.

Some of the known vitamins seem to influence the production of the anemia. For example there is some reason to believe the incidence is higher when pyridoxine is supplied at a low level. Presumably this vitamin does not exert a specific effect, but it is possible that it aids in the bacterial synthesis of the antianemia vitamin in the intestine.

Evidence is presented to show that the factor is transmitted through the egg to the chick and that it is more abundant in green than in dry feeds. For this reason it is advantageous to restrict the hatching flock to dry feeds only, in order to obtain more suitable experimental chicks.

The recent work of Black, Overman, Elvehjem, and Link (15), in which they fed sulfaguanidine to rats, shows that there is another factor required by the rat when bacterial synthesis in the intestine is inhibited. Our data show that the chick develops anemia more readily when sulfaguanidine is supplied in the diet. However, since sulfaguanidine may be slightly toxic, it is preferable to omit it if a suitable diet can be formulated that does not

[5] This fraction was kindly supplied by Dr. Y. Subbarow.

contain the drug. Such a diet can be prepared by including wheat germ oil as a source of fat, by adding biotin to the vitamin combination, and by omitting the alcoholic extract of liver.

The properties of the substance described by Black *et al.* (7) as being required by the rat are very similar to those exhibited by vitamin B_c. The properties of the antianemia vitamin as indicated by its adsorption behavior and by salt formation with various bases indicate an acid. The precipitation with phosphotungstic acid indicates basic properties, although it is possible that the factor is merely adsorbed on the precipitate formed by the phosphotungstic acid. The factor is very labile to acid, but somewhat more stable toward alkali. It is not easily destroyed by oxidation. One of its salient characteristics is insolubility in the common organic solvents.

Mills, Briggs, Elvehjem, and Hart (3) have called attention to the similarity in the properties of the eluate fraction required by *Lactobacillus casei* ϵ, the factor U of Stockstad and Manning (16), folic acid described by Mitchell, Snell, and Williams (17), and vitamin B_c. The properties of the factor required for lactic acid bacteria, which was described by Hutchings, Bohonos, and Peterson (18), are very similar to those of vitamin B_c in all points investigated. Both factors are quite labile to acid, are precipitated by heavy metals, are adsorbed on fullers' earth and norit, and are insoluble in the common organic solvents.

SUMMARY

1. A diet has been developed which will produce a severe anemia in at least 50 per cent of the chicks.

2. A technique has been devised for the assay of the factor, essential in chick nutrition, which prevents the anemia.

3. Sulfaguanidine increased the incidence of anemia under the experimental conditions imposed at that time.

4. Vitamin B_c is acidic in nature and forms salts with heavy metals. It is destroyed by mineral acids (pH 1) but is more stable to alkali. It is adsorbed from acidic solution by a variety of adsorbents and can be eluted by ammonia. It is insoluble in the common organic solvents.

BIBLIOGRAPHY

1. Hogan, A. G., and Parrott, E. M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. xlvi (1939).
2. Hogan, A. G., and Parrott, E. M., *J. Biol. Chem.*, **132**, 507 (1940).
3. Mills, R. C., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **49**, 186 (1942).
4. Van Allen, C. M., *J. Lab. and Clin. Med.*, **10**, 1027 (1925).
5. Mills, F. C., Statistical methods, New York, 355 (1938).

6. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, **14**, 273 (1937).
7. Black, S., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **47**, 308 (1941).
8. Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, **134**, 213 (1940).
9. Hegsted, D. M., Hier, S. W., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **139**, 863 (1941).
10. Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404 (1943).
11. Ungley, C. C., *Lancet*, **2**, 1513 (1936).
12. Myers, R. J., Eastes, J. W., and Myers, F. J., *Ind. and Eng. Chem.*, **33**, 697 (1941).
13. Simmons, R. W., and Norris, E. R., *J. Biol. Chem.*, **140**, 679 (1941).
14. Purrmann, R., *Ann. Chem.*, **546**, 98 (1941).
15. Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, **145**, 137 (1942).
16. Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **125**, 687 (1938).
17. Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, **63**, 2284 (1941).
18. Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

STABILITY OF THIAMINE TO HEAT

I. EFFECT OF pH AND BUFFER SALTS IN AQUEOUS SOLUTIONS*

By B. W. BEADLE, D. A. GREENWOOD, AND H. R. KRAYBILL

(From the Research Laboratory, American Meat Institute, University of Chicago, Chicago)

(Received for publication, May 3, 1943)

It has been known for some time that thiamine is less stable to heat in alkaline solution than in acid solution ((1) p. 104, (2)). Solutions of thiamine at pH 3 and 6 are said to be unaffected on heating for 30 minutes at 100° (2). They are reported to be unaffected by sterilizing at pH 3.5 for 1 hour at 100°, or 20 minutes at 120° (2). Sherman and Burton (3) found that tomato juice lost 20 per cent of its thiamine content during 4 hours heating at 100° at the natural pH of 4.28, but when the pH was raised to 9.2, the destruction was 60 to 70 per cent in 1 hour. Keenan, Kline, Elvehjem, and Hart (4) showed that vitamin B_1 in yeast, liver, and in a natural grain ration was inactivated completely by autoclaving for 5 hours. Eddy, Kohman, and Carlsson (5) stated that no cooking or canning process affects appreciably the content of vitamins A and B in green peas, while the Medical Research Council stated in a special report (6) that canned foods of all descriptions may contain very little or no vitamin B_1.

It is apparent from the above statements that there are factors involved in the destruction of thiamine by heat which are little understood, since losses of thiamine may occur in one food but not in another, during similar processing, and since different workers draw different conclusions from experimental data on the effects of processing of foods. As an aid to the more complete understanding of the problem it is of interest to make a systematic study of the behavior of pure thiamine at various pH values in different buffer solutions. Such a study should indicate whether or not the destruction of pure thiamine due to heat is a factor of pH only, or whether the buffer system itself is of importance in determining the amount of destruction. The purpose of this paper is to present the results of such a study as were obtained in a series of experiments with pure thiamine.

EXPERIMENTAL

The pH values of the solutions were determined by means of a Leeds and Northrup type 7661-A1 instrument, with which it is easily possible to

* Presented before the Division of Agricultural and Food Chemistry at the One-hundred-fifth meeting of the American Chemical Society at Detroit, April 13, 1943.

reproduce values to within 0.05 pH unit. All solutions were subjected to pH determination both before and after treatment.

The thiamine assays were made by the thiochrome procedure for estimation of thiamine, as outlined by the Research Corporation Committee (7) and by spectrophotometric examination of the ultraviolet absorption spectra. The spectrophotometric studies were conducted by means of a model DU Beckman photoelectric spectrophotometer, having quartz optics, with a hydrogen discharge tube as the source of continuous radiation in the ultraviolet.

The absorption spectrum of thiamine is known to be affected by the pH of the medium ((1) p. 103, (8–14)). It was found, however, that the ab-

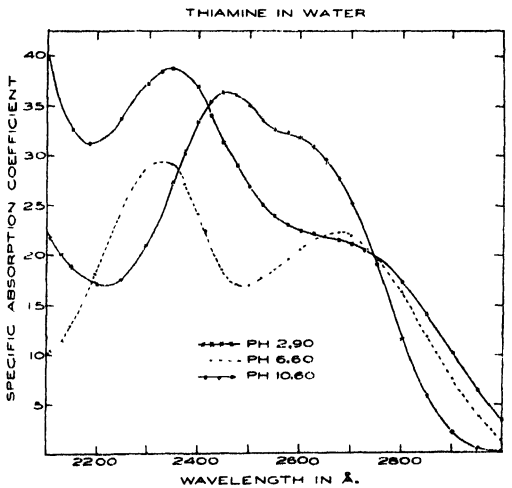


FIG. 1

sorption curve is so radically affected by small changes in pH that duplication of data was not possible unless the pH values were controlled very closely, to about 0.05 pH unit. Fig. 1 shows typical absorption data obtained for aqueous solutions of pure thiamine chloride hydrochloride as supplied by Merck and Company, Inc. (in the remainder of this paper the term "thiamine" refers to thiamine chloride hydrochloride). The specific absorption coefficients were calculated from the Lambert-Beer equation (15), $\log I_0/I = \alpha cl$, where I_0 = the intensity of radiation transmitted by the solvent, I = the intensity of radiation transmitted by the solution, α = the specific absorption coefficient, c = the concentration in gm. per liter, and l = the length of solution in cm.

At the wave-length 2600 Å. (an analytical point selected by the authors for analysis of binary systems in another study), the relation between the

specific absorption coefficient and the pH of the aqueous solution was studied. Fig. 2 shows this relationship graphically, between pH 1 and 11. Through the use of this curve, the specific absorption coefficient may be readily learned for the pH values of the solutions being examined. It may be seen in Fig. 2 that the absorption coefficient at this wave-length decreases rapidly as the pH rises from 3 to 5.5, remains fairly constant between pH 5.5 and 7.5, then increases rapidly as the pH rises from 7.5 to 10.

The solutions of thiamine used throughout the series of studies were prepared by the addition of 10 ml. portions of a stock solution, containing 100 γ of pure thiamine per ml., to 100 ml. volumetric flasks. The flasks were then filled almost to the mark with water (or the aqueous buffer

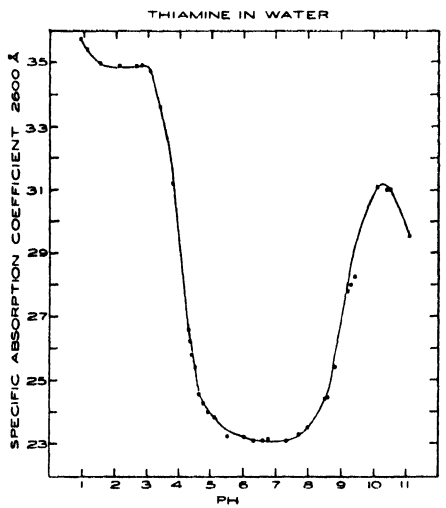


FIG. 2

solution), final adjustment of pH made, and the flasks filled to the mark. This procedure yielded solutions containing 10 γ of thiamine per ml., a concentration well within the range of that reported in many meats and other foodstuffs. Aliquots of these solutions were then placed in smaller flasks and immersed in boiling water for the desired time, after which they were cooled rapidly to room temperature. Spectrophotometric examinations and chemical analyses were then made on aliquots of the solutions both before and after the heating period.

The unbuffered solutions were brought to the desired pH by the addition of 0.01 N sodium hydroxide or 0.01 N hydrochloric acid, as required. At extreme pH values more concentrated reagents were required. The pH of thiamine in distilled water at a concentration of 10 γ per ml. is about 4.25.

The buffer solutions were prepared according to the so called standard

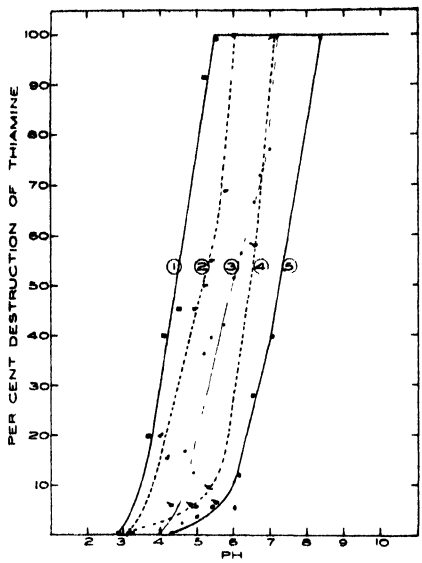


FIG. 3

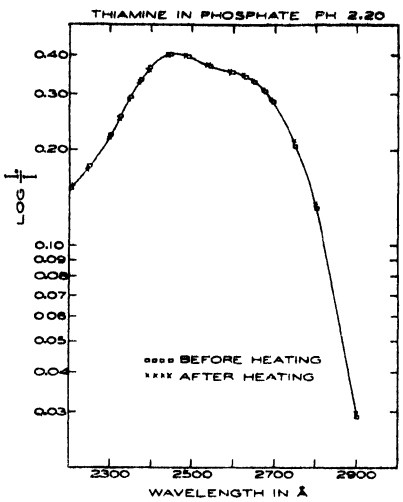


FIG. 4

FIG. 3. Effect of heat on thiamine in solution. Curve 1 represents borate buffer; Curve 2, unbuffered solution after heating for 60 minutes; Curve 3, unbuffered solution after heating for 30 minutes; Curve 4, acetate buffer; Curve 5, phosphate buffer.

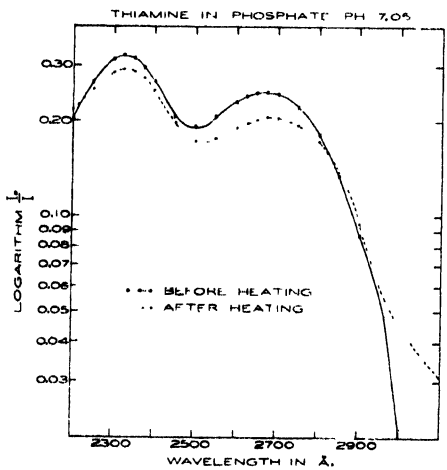


FIG. 5

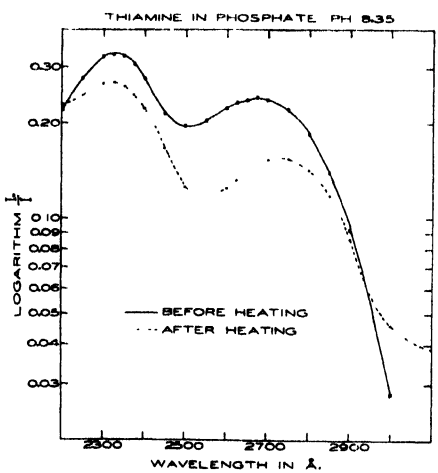


FIG. 6

methods (16, 17). The acetate solutions were those of Walpole (17), the borate solutions were those of Palitzsch (17), and the phosphate solutions those of Sørensen (16). At pH values outside the ranges covered by the

buffer systems, sodium hydroxide or hydrochloric acid was added to obtain the desired pH in the presence of the buffer salts.

Fig. 3 is a graphic portrayal of the results obtained in unbuffered solution after heating in boiling water for periods of 30 minutes and 60 minutes, respectively, and in the buffer solutions after a heating period of 60 minutes.

Figs. 4, 5, and 6 present typical spectrophotometric data obtained on the solutions before and after the heating periods. While the curves in these figures were obtained on phosphate solutions, the same results were obtained in the unbuffered series and in the borate series. Lack of space does not justify presenting all of them. It was not practical to attempt spectrophotometric interpretation of the acetate solutions owing to strong absorption by the acetate ion at wave-lengths shorter than 2500 Å. All the absorption spectra except those of Fig. 1 are plotted with the logarithm of log I_0/I as the ordinate, and wave-length in Å. as the abscissa, for the purpose of ease in comparison. Changes in concentration result merely in moving the curve to a different height on the ordinate, without altering its shape (18). Absorption measurements were reproducible within about 1 per cent.

DISCUSSION

The data presented in this report represent nearly 200 thiamine analyses. While an occasional analysis failed to fall exactly on the curves shown in Fig. 3, such instances were rare and are believed by the authors to be due to inherent errors in the thiochrome method or the technique of the operator. It was estimated as the result of analyses on known solutions that individual results may vary about 5 per cent from the known, or a total of about 10 per cent between the extremes. As is evidenced by Fig. 3, all of the curves are so steep that the pH effect in each instance is far greater than this, even within rather narrow limits. The destruction of thiamine rises from 0 to 100 per cent within 2 or 3 pH units.

The effect of variations in the electrolyte system at a given pH on the stability of thiamine to heat is striking. For example, at pH 5.4, as shown in Fig. 3, there was 100 per cent destruction of the vitamin during 1 hour's heating in the presence of borates, 60 per cent destruction in unbuffered aqueous solution, 10 per cent in the presence of acetates, and about 3 per cent in the phosphate solution. At pH 7, there was 100 per cent destruction in the unbuffered solution and in the borate and acetate solutions, while only 40 per cent of the thiamine was destroyed under similar conditions in the phosphate solution. The effect of time on destruction of thiamine in unbuffered solution is illustrated by the figures at pH 6.0. All the vitamin was destroyed during 1 hour's heating, while about 50 per cent was destroyed in the 30 minute period. When these samples

were allowed to stand for as long as 10 days in the refrigerator (5°) without going through the heating period, no losses were observed either in the unbuffered or the buffered solutions.

It can hardly be concluded from the above that the destruction is simply a salt effect, for in this instance the amount of destruction should have been the least in the unbuffered solutions, where the least amount of salt was present.

A quantitative comparison of the spectrophotometric data on thiamine presented in Fig. 1 with the values in the literature cannot be made in most instances, owing to difficulty in interpolating the values published graphically and also to lack of sufficient data regarding the pH and solvent.

Melnick (14) has published molecular extinction coefficients for thiamine at pH 7.4 in phosphate buffer solution, using a Bausch and Lomb medium spectrograph. His values were 10,250 and 6000 at the maxima 2350 and 2650 Å., respectively. The values obtained in this laboratory for the thiamine used in these studies with the photoelectric spectrophotometer were 10,843 and 8383 at the above wave-lengths, at pH 7.4, in Sørensen's phosphate solution. Values obtained on a sample of U.S.P. reference standard thiamine were 10,885 and 8290 under the same conditions.

There is considerable evidence that chemical methods of assay for thiamine are not completely reliable ((1) p. 128–130); therefore, one may well suspect that the ions present in the various buffer series encountered in these experiments may have interfered with the thiochrome procedure, thereby vitiating the conclusions regarding the losses in the various systems. The spectrophotometric examinations typified in Figs. 4, 5, and 6 were made as a means of establishing whether or not destruction of thiamine actually occurred when indicated by the thiochrome assay. No instances were found in which the spectrophotometric data were contrary to those obtained by the thiochrome method. When no destruction of thiamine was indicated by the thiochrome method, the absorption curves from solutions before and after heating were perfectly superposable, matching at all points, as shown in Fig. 4. When losses were found by the thiochrome method, the absorption curves indicated the destruction by shifts in the curve both as to height and points of maxima, as shown in Figs. 5 and 6. The greatest changes in the absorption spectra were found in those solutions in which destruction was greatest, as determined by the thiochrome procedure. This indicates that under the conditions encountered the thiochrome method was reliable as an index of the thiamine present. No bioassays were made.

It is interesting to note that in all instances in which complete destruction of thiamine occurred the resulting solutions yielded characteristic absorption spectra, as shown in Fig. 6. The fact that these spectra closely resemble

the spectra of compounds of the pyrimidine type similar to the pyrimidine nucleus of thiamine leads one to the conclusion that the destruction in all the instances investigated did not break down the pyrimidine component of the molecule. It has been stated that sulfites cause cleavage of the thiamine molecule (19, 20), also that barium nitrite and possibly sodium acetate cause this same cleavage (21, 22). The authors found the absorption characteristics mentioned above in all the buffers examined, as well as in the unbuffered solution to which no salts had been added but in which complete destruction of the thiamine was indicated. While it must be remembered that the absorption curve shown in Fig. 6 is probably complicated by the thiazole residues, its similarity to the pyrimidine curves of Uber and Ver-

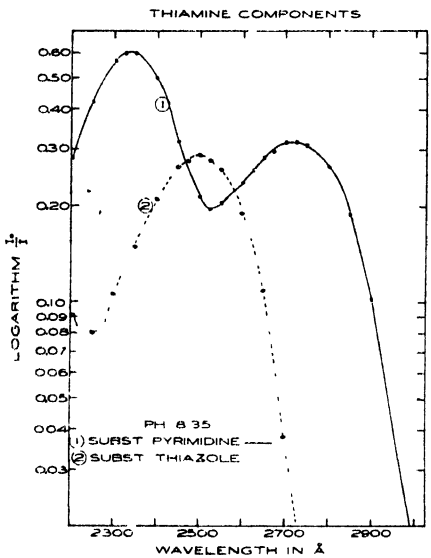


FIG. 7

brugge (13) is interesting. Through the courtesy of Dr. Randolph T. Major of Merck and Company, Inc., the authors obtained samples of the substituted pyrimidine and thiazole compounds from which thiamine is synthesized. The absorption spectrum of the pyrimidine component in phosphate buffer at pH 8.3 is shown in Fig. 7, together with the spectrum of the thiazole. The similarity of the pyrimidine curve to the lower curve in Fig. 6 is apparent. The sulfite cleavage products have been shown to possess no vitamin B_1 activity (19, 20). But more recent studies (23, 24) indicate that synthesis of thiamine from the cleavage products may occur in the digestive tract.

The data presented here, while not complete in the sense of covering all

natural buffers, indicate the need for caution in making generalizations in regard to the stability of thiamine under various conditions of processing. The varying results reported by workers on different foods may quite possibly be due not only to variations in pH within the tissues, but also to variations in the electrolyte systems and possibly to other factors such as the protein systems involved. It has been noted that thiamine is more stable in biological tissues than in pure solution (25). Williams (21) has suggested that possibly cocarboxylase (the pyrophosphoric acid ester of thiamine) may exhibit a stability to heat different from that of pure thiamine. Greenwood, Beadle, and Kraybill (26) have found that certain proteins exert a strong protecting action on thiamine, and that cocarboxylase is only slightly more stable to heat than is thiamine.

SUMMARY

Results of chemical and spectrophotometric examination of nearly 200 solutions of pure thiamine indicate that the stability of thiamine to heat is a function not only of pH but also of the electrolyte system involved. At pH 5.4, during 1 hour's heating in boiling water there was 100 per cent destruction of the thiamine in the presence of borates, 57 per cent destruction in unbuffered aqueous solution, 10 per cent destruction in the presence of acetates, and 3 per cent in phosphate solution. In each type of solution, destruction rose from 0 to 100 per cent within the range of 2 to 3 pH units during 1 hour's heating period.

BIBLIOGRAPHY

1. Rosenberg, H. R., Chemistry and physiology of the vitamins, New York (1942)
2. Addinall, C. R., The story of vitamin B_1, Rahway, 17 (1940).
3. Sherman, H. C., and Burton, G. W., *J. Biol. Chem.*, **70**, 639 (1926).
4. Keenan, J. A., Kline, O. L., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **9**, 63 (1935)
5. Eddy, W. H., Kohman, E. F., and Carlsson, V., *Ind. and Eng. Chem.*, **18**, 85 (1926).
6. *Med. Research Council, Special Rep. Series, No. 167*, 144 (1932).
7. Hennessy, D. J., *Cereal Chem. Bull.*, **2**, No. 2 (1942).
8. Wintersteiner, O., Williams, R. R., and Ruehle, A. E., *J. Am. Chem. Soc.*, **57**, 517 (1935).
9. Smakula, A., *Z. physiol. Chem.*, **230**, 231 (1934).
10. Peters, R. A., and Philpot, J. St. L., *Proc. Roy. Soc. London, Series B*, **113**, 48 (1933).
11. Holiday, E. R., *Biochem. J.*, **29**, 718 (1935).
12. Williams, R. R., Ruehle, A. E., and Finkelstein, J., *J. Am. Chem. Soc.*, **59**, 526 (1937).
13. Uber, F. M., and Verbrugge, F., *J. Biol. Chem.*, **134**, 273 (1940).
14. Melnick, J. L., *J. Biol. Chem.*, **131**, 615 (1939).
15. Hogness, T. R., and Potter, V. R., Annual review of biochemistry, Stanford University, **10**, 509 (1941).

16. Gortner, R. A., Outlines of biochemistry, New York, 2nd edition, 124 (1938).
17. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 115 (1925).
18. Strain, H. H., Leaf xanthophylls, Washington (1938).
19. Williams, R. R., *J. Am. Chem. Soc.*, **57**, 229 (1935).
20. Williams, R. R., Waterman, R. E., Keresztesy, J. C., and Buchman, E. R., *J. Am. Chem. Soc.*, **57**, 536 (1935).
21. Williams, R. R., *J. Am. Med. Assn.*, **110**, 727 (1938).
22. The vitamins, American Medical Association, Chicago, 149, 150 (1939).
23. Black, S., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **47**, 308 (1941).
24. Mitchell, H. K., and Isbell, E. R., *Univ. Texas Pub., No. 4237*, 125 (1942).
25. Melnick, D., Robinson, W. D., and Field, H., Jr., *J. Biol. Chem.*, **138**, 49 (1941).
26. Greenwood, D. A., Beadle, B. W., and Kraybill, H. R., *J. Biol. Chem.*, **149**, 349 (1943).

STABILITY OF THIAMINE TO HEAT

II. EFFECT OF MEAT-CURING INGREDIENTS IN AQUEOUS SOLUTIONS AND IN MEAT*

By D. A. GREENWOOD, B. W. BEADLE, AND H. R. KRAYBILL

(From the Research Laboratory, American Meat Institute, University of Chicago, Chicago)

(Received for publication, May 3, 1943)

Meats, especially lean pork, constitute one of the important natural sources of thiamine for man. Cured meats, in addition to fresh meats, are consumed in large quantities by men in the armed forces and by civilians. The vitamin content of foods in relation to processing and other variants has been summarized recently by Booher, Hartzler, and Hewston (1), investigators at the University of Texas Biochemical Institute (2), Waisman and Elvehjem (3), and Fixsen (4).

Melnick, Robinson, and Field (5) have studied the effect of digestive juices on the stability of thiamine. They emphasized the point that thiamine is more stable in its natural environment than in pure aqueous solutions.

The purpose of this paper is to report the effect on thiamine of heating aqueous thiamine solutions and meat in the presence of the substances commonly used in the curing of meats (sodium chloride, sodium nitrate, sodium nitrite, sucrose, and dextrose).

EXPERIMENTAL

Effect of Heat on Loss of Thiamine in Aqueous Solution

Aqueous solutions of U.S.P. thiamine chloride hydrochloride, sodium chloride, sodium nitrate, sodium nitrite, sucrose, and dextrose were prepared in the concentrations found in cured meats. The concentration of sodium chloride in most cured meats varies from 3 to 5 per cent. The present trend is toward a mildly cured product with a salt content of about 3 per cent. In some cases, excessive amounts of curing substances were added to the thiamine solutions. The United States Bureau of Animal Industry limits the concentration of sodium nitrite to 0.02 per cent in cured meats sold in interstate commerce. Most of the cured meats offered to the public contain much less than 0.02 per cent sodium nitrite. Some of the solutions were heated in unbuffered solutions, while others were buffered at different hydrogen ion concentrations by Sørensen's phosphate mixtures

* Presented before the Division of Agricultural and Food Chemistry at the One-hundred-fifth meeting of the American Chemical Society at Detroit, April 13, 1943.

as described by Gortner (6). The pH measurements were obtained with a Leeds and Northrup electrometer, No. 7661-A1, and glass electrode.

The samples were heated in 50 or 100 ml. flasks by immersing in a boiling water bath (98°) for various time intervals, after which they were immediately placed in cold water (4–10°).

Thiamine determinations were made by the thiochrome method described by the Research Corporation Committee (7). In most cases the

TABLE I

Effect of Heating Aqueous Solutions of Thiamine (10 γ per Ml.) in Boiling Water Bath (98°) for 1 Hour in Presence of Meat-Curing Ingredients

All analyses were made in duplicate. Samples 6 to 18 were buffered at pH 5.60 or 6.10 by the addition of appropriate volumes of Sørensen's phosphate mixtures and sodium hydroxide

Sample No.	Sodium chloride	Sodium nitrate	Sodium nitrite	Sucrose	Dextrose	pH of solution	Loss	Loss at pH 6.10
	per cent	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*
1						4.10	0.0	
2	1.0					4.70	19.0	
3	3.0					4.65	31.0	
4	4.0					4 65	31.0	
5	5.0					4 65	31 0	
6						5.60	0.0	0.0
6a	0.1							2.0
6b	1.0							93.0
7	3.0					5.60	81.0	100.0
8	4.0					5.60	80.0	100 0
9	5 0					5.60	81 0	
10		0.05				5 60	3.0	0.0
11			0.02			5.60	12.0	34.5
12			0.10			5.60	62 0	69.0
13				0 5		5.60	0.0	0.0
14					0.5	5.60	0.0	0.0
15	3.0	0.05	0 02	0 5		5.60	82.0	96.5
16	3.0	0 05	0.02		0 5	5.60	81.0	96.5
17	3.0	0.05	0.10	0 5		5.60	81.0	100.0
18	3 0	0.05	0.10		0 5	5 60	82.0	100 0

chemical results were duplicated with an error of less than ±1 per cent. The maximum variation in duplicate determinations was ±5 per cent. The analytical error commonly accepted for the thiochrome method is from ±5 to ±10 per cent. Losses of thiamine greater than 10 per cent may be considered greater than the analytical error involved in the thiochrome method.

Absorption spectra studies, similar to those reported in Paper I (8), were made on some of the aqueous thiamine solutions before and after heating.

A Beckman quartz spectrophotometer model DU was used in obtaining the spectrophotometric data. When losses were indicated by the thiochrome method, the spectrophotometric data also indicated similar losses except in cases in which substances were present which interfered with the spectrophotometric measurements.

Table I contains data which show the effect of heating aqueous solutions of thiamine for 1 hour in the presence of various concentrations of sodium chloride, sodium nitrate, sodium nitrite, sucrose, and dextrose. Attention is directed to the significant destruction (19 to 100 per cent) of thiamine which occurred when aqueous solutions of the vitamin were heated in the presence of sodium chloride in unbuffered and phosphate-buffered solutions.

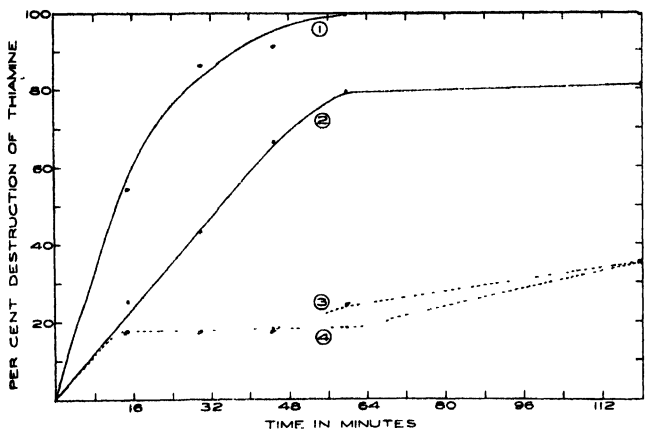


FIG. 1. The effect of the length of heating time in boiling water (98°) on the destruction of thiamine in aqueous solutions. Curve 1 represents 10 γ per ml. of thiamine plus the following substances in per cent: sodium chloride 3, sodium nitrate 0.05, sodium nitrite 0.02, sucrose 0.5, and Sørensen's phosphate mixtures and sodium hydroxide to give pH 6.10 ± 0.05; Curve 2, same as Curve 1 except that the pH was adjusted to 5.60 instead of 6.10; Curve 3, same as Curve 1 except that 2 per cent egg albumin was added; Curve 4, same as Curve 2 except that 2 per cent egg albumin was added.

The destruction of thiamine in the presence of curing substances is a function of the concentration of the curing agents (except sodium nitrate, sucrose, and dextrose) and the pH of the medium. Studies were made at pH values of 5.60 and 6.1, because these levels represent the hydrogen ion concentrations frequently encountered in fresh and cured meats.

A significant loss (12 to 69 per cent) of thiamine occurred when the vitamin was heated in aqueous solution in the presence of 0.02 to 0.10 per cent sodium nitrite. Williams (9) stated that barium nitrite would cause a cleavage of thiamine but he did not give the exact experimental conditions under which cleavage occurred.

Destruction of thiamine during heating in aqueous phosphate buffer solutions was about the same in the presence of combinations of the curing substances as in 3 per cent sodium chloride.

Another part of the study dealt with the influence of the length of heating period in boiling water (98°) upon the destruction of thiamine in the presence of the curing ingredients, egg albumin, and Sørensen's phosphate mixtures, as shown in Fig. 1. The results indicate that destruction of thiamine increased with the length of the heating period. The greatest loss of thiamine occurred when the vitamin was heated in the presence of the curing ingredients and Sørensen's phosphate mixtures at pH 6.1. Most of the thiamine was destroyed during the first 30 minutes of heating under these conditions. On the other hand, the thiamine was most stable when it was heated in the presence of 2 per cent egg albumin, curing ingredients, and Sørensen's phosphate mixtures at pH 5.6. Comparable results (not shown in Fig. 1) were obtained when thiamine solutions were heated under similar conditions except that gelatin was substituted for egg albumin.

Effect of Heat on Loss of Thiamine in Lean Pork

A portion of the study was concerned with the effect of heating thiamine as it occurs in meat in the presence of meat-curing substances. The lean portion of about a kilo of fresh pork chops was ground three times in a meat grinder and mixed thoroughly. To samples of this ground lean pork were added various amounts and combinations of the meat-curing substances.

Test-tubes (150 × 14 mm.) containing samples of ground meat, mixed thoroughly with the curing ingredients, were immediately heated in a boiling water bath (98°) for 1 hour and then cooled promptly in a cold running water bath (4–10°). Other samples containing curing substances were stored for 10 days in a refrigerator at 0–2°. They were then heated for 1 hour in a boiling water bath (98°) and then cooled.

Table II contains data relating to the effect on thiamine content of heating pork muscle for 1 hour at 98° in the presence of different combinations and amounts of curing substances. The results indicate that destruction of thiamine in pork muscle under the conditions of these experiments was about the same in the presence and absence of meat-curing substances. While there were slight differences in the thiamine losses in different samples, the variations were within the limits of experimental error involved in the thiochrome method for determination of thiamine.

The losses of thiamine, when heated in the presence of pork muscle, were in marked contrast to the losses which occurred in pure thiamine solutions in which 19 to 100 per cent of the vitamin was destroyed when such solutions were heated for 1 hour in a boiling water bath (98°).

The effect of heating on the thiamine content of pork muscle which had

been stored for 10 days in the presence of curing substances is also given in Table II. The data indicate that destruction of thiamine upon heating the meat is about the same whether or not the curing ingredients are present.

The destruction of thiamine in the meat during heating for 1 hour at 98° varies from 18 to 21.1 per cent.

Our observations regarding the stability of thiamine confirm the findings of Melnick, Robinson, and Field (5); namely, that the vitamin is more stable in its natural environment than in aqueous solutions.

TABLE II

Effect of Heating Lean Pork Muscle in Boiling Water Bath (98°) for 1 Hour in Presence of Substances Used in Curing of Meats

All analyses were made in duplicate. pH of samples, 6.00 ± 0.02.

Heated immediately after addition of curing ingredients						Heated after storing for 10 days at 0–2° in presence of curing ingredients	
Sample No.	Sodium chloride	Sodium nitrate	Sodium nitrite	Thiamine	Heating loss	Thiamine	Heating loss
	per cent	*per cent*	*per cent*	*γ per gm.*	*per cent*	*γ per gm.*	*per cent*
1, 13*				19.4		19.4	
2, 14				16.2	16.5	15.9	18.0
3, 15	1.0			16.2	16.5	15.9	18.0
4, 16	2.0			16.2	16.5	15.9	18 0
5, 17	3.0			16.2	16.5	15.9	18.0
6, 18	4.0			16.2	16.5	15.9	18.0
7, 19	5 0			16.2	16.5	15.9	18.0
8, 20		0 05		16.7	13 9	15.3	21.1
9, 21			0.10	15.9	18.0	15.6	19.6
10, 22			0.02	16.0	17.5	15.3	21.1
11, 23	3 0	0.05	0.10	15.9	18.0	15.3	21.1
12, 24	3.0	0.05	0.02	16.1	17.0	15.6	19.6

* Samples 1 and 13 were not heated.

In Paper I (8), data are presented which indicate that aqueous solutions of thiamine are more stable in phosphate than in acetate or borate buffers at the same pH values. Experiments were therefore conducted on cocarboxylase (Merck's). Aqueous solutions of cocarboxylase (10 γ per ml.) were heated for 1 hour in a boiling water bath (98°) in the presence of 1 to 3 per cent sodium chloride, 0.05 per cent sodium nitrate, and 0.02 per cent sodium nitrite. Some of the solutions were adjusted to pH values of 5.6 and 6.1 with Sørensen's phosphate buffer mixtures and sodium hydroxide. The losses of cocarboxylase under these conditions varied from 19 to 72 per cent. When aqueous solutions of thiamine were heated under comparable conditions, the losses varied from 19 to 100 per cent. These experiments indicate

that cocarboxylase is slightly more stable than thiamine under the same experimental conditions. On the other hand, the destruction of cocarboxylase which occurs upon heating resembles the behavior of aqueous solutions of thiamine more closely than the thiamine of pork muscle.

Our experiments dealing with the stability of thiamine indicate that the presence of several substances both inorganic and organic alter the stability of the vitamin during heating. Great caution should be exercised in projecting data from one set of experimental conditions to different conditions.

SUMMARY

The effects of heating aqueous solutions of thiamine and cocarboxylase and meat in the presence of the substances commonly used in curing meats (sodium chloride, sodium nitrate, sodium nitrite, sucrose, and dextrose) on the loss of thiamine are reported. The losses varied from 0.0 to 100 per cent. The destruction of the thiamine upon heating is a function of the temperature, time of heating, the pH of the medium, and the nature of the other substances present. Thiamine is less stable to heat in aqueous solutions than in lean pork muscle.

There was no significant difference in the loss of thiamine when lean pork was heated (1 hour at 98°) in the presence or absence of meat-curing ingredients, or in the loss of thiamine in fresh lean pork and cured (10 days) lean pork when heated for 1 hour at 98°. The loss in thiamine during heating of lean pork in the absence of curing ingredients ranged from 16.5 to 18.0 per cent and in the presence of curing ingredients from 16.5 to 21.1 per cent.

BIBLIOGRAPHY

1. Booher, L. E., Hartzler, E. R., and Hewston, E. M., *U. S. Dept. Agric., Circ. 638* (1942).
2. *Univ. Texas Pub., No. 4137* (1941); *No. 4237* (1942).
3. Waisman, H. A., and Elvehjem, C. A., The vitamin content of meat, Minneapolis (1941).
4. Fixsen, M. A., *Nutrition Abst. and Rev.*, **8**, 281 (1938).
5. Melnick, D., Robinson, W. D., and Field, H., Jr., *J. Biol. Chem.*, **138**, 49 (1941).
6. Gortner, R. A., Outlines of biochemistry, New York, 2nd edition, 124 (1938).
7. Hennessy, D. J., *Cereal Chem. Bull.*, **2**, No. 2 (1942).
8. Beadle, B. W., Greenwood, D. A., and Kraybill, H. R., *J. Biol. Chem.*, **149**, 339 (1943).
9. The vitamins, American Medical Association, Chicago, 149, 150 (1939).

THE RELATION OF TRANSMETHYLATION TO ANSERINE

By JAY R. SCHENCK,* SOFIA SIMMONDS, MILDRED COHN, CARL M. STEVENS, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, May 27, 1943)

In a previous communication (1), it was reported that the methyl group of dietary methionine was utilized by the rat for the synthesis of choline, creatine, and creatinine. It was desirable, as we had previously pointed out (1, 2), to investigate the possibility that methionine could also serve as the source of the methyl group of other methylated compounds in the animal body. When the transmethylation studies were extended to the rabbit, it became feasible to isolate in sufficient quantities one of the N-methyl extractives; namely, anserine. It thus became possible to determine by the isotope technique whether a transfer of methyl groups from methionine to anserine could occur.

Before an attempt was made to relate anserine to the transmethylation reactions, it was first necessary to demonstrate that the methyl transfer which occurs in the rat also proceeds in the rabbit. Therefore, deuteriomethionine corresponding to 0.5 per cent of the diet was fed to a rabbit with fibrin as the source of protein in the diet and the creatinine was isolated from the 7th and 8th day samples of urine. The creatinine was found to contain deuterium in an amount which indicated that 4 per cent of the methyl groups had been derived from the deuteriomethyl groups of the diet. Since this value was rather low, it was deemed advisable to increase the dietary deuteriomethionine to 1 per cent of the diet. Subsequently creatinine was isolated from the urine of the 17th and 18th days and again from the urine of the 25th and 26th days, indicating that 12 per cent and 20 per cent respectively of the methyl groups of creatinine had been derived from the ingested deuteriomethionine. The animal was sacrificed after 28 days and the anserine, creatine, and choline were isolated from the tissues.

The presence of deuterium in the choline and creatine demonstrated that the rabbit as well as the rat utilized the methyl group of methionine for the synthesis of choline and creatine. The deuterium content of the methyl groups of the isolated choline was lower than that of the creatine. This result was undoubtedly due, in part, to the presence of ordinary choline in

* Abbott Laboratories Research Fellow.

the diet. The "bound" choline and "free" choline fractions differed considerably in deuterium content.[1]

The anserine which was isolated from the muscle tissues was found to contain deuterium in an amount which indicated that 2 per cent of the total anserine had been derived from the ingested deuteriomethionine during the experimental period. Although the conversion from methionine to anserine is apparently much slower than from methionine to creatinine, the amount of deuterium found in the isolated anserine is unquestionably far beyond the experimental error.

EXPERIMENTAL

Feeding Experiment—A rabbit weighing 1.9 kilos was fed *ad libitum* a basal diet consisting of the following components: fibrin 10 per cent, sucrose 36 per cent, starch 21 per cent, corn oil (Mazola) 14 per cent, agar 10 per cent, yeast 5 per cent,[2] salt mixture (3) 3.5 per cent, choline chloride 0.1 per cent. The fat-soluble vitamins at the same level as used in the rat diets (4) and also 200 mg. of ascorbic acid per kilo were added to the diet in the latter half of the experimental period. For the first 12 days the diet was supplemented with 0.5 per cent deuteriomethionine containing 21.6 atom per cent deuterium; *i.e.*, 79 atom per cent in the methyl group. Since only 4.3 per cent of the methyl groups of urinary creatinine had been derived from deuteriomethionine after 8 days, as shown in Table I, the deuteriomethionine in the diet was increased to 1 per cent of the total diet on the 12th day. The urine was collected over 48 hour periods at intervals during the experiment. The deuterium concentrations of the creatinine of the urine for the 17th and 18th days and for the 25th and 26th days are shown in Table I. The animal was sacrificed after 28 days and the anserine, the choline, and the creatine were isolated. The deuterium concentrations of the isolated compounds are given in Table II.

Isolation of Anserine—The muscle tissue (595 gm.) was extracted with sodium sulfate in the manner described by Deutsch, Eggleton, and Eggleton (5). This method is applicable for the isolation of anserine in this case, since the amount of anserine far exceeds that of carnosine in rabbit muscle tissue. The copper salt obtained by this method was extracted with absolute methyl alcohol from inorganic salts.

[1] In some unpublished experiments with rats, it has been found that the "bound" and "free" choline fractions differed in the same manner as in the present experiments. Also, when ordinary choline is fed to rats whose diets contain deuteriomethionine, the deuterium content of creatine isolated from the tissues is higher than the deuterium content of the tissue choline, analogous to the observations with the rabbit.

[2] Anheuser-Busch Strain G dried brewers' yeast, Biological Laboratory No. 1820, Blend D.

After evaporation of the methyl alcohol, the copper salt was dissolved in dilute sulfuric acid and the copper was subsequently removed by precipitation with hydrogen sulfide. The precipitate was filtered and the filtrate was concentrated to a small volume (17 cc.). 35 cc. of Hopkins' reagent (6) were added portionwise to the solution; at the same time, sufficient absolute ethyl alcohol (124 cc.) was added to maintain a concentration

TABLE I

*Creatinine from Urine of Rabbit Fed Deuteriomethionine**

Interval of urine collection	Creatinine isolated		B/A† × 100
	Deuterium in creatinine K picrate	Deuterium in methyl group (B)	
days	*atom per cent*	*atom per cent*	
7– 8	0.86 ± 0.06	3.4	4.3
17–18	2.36 ± 0.06	9.4	11.9
25–26	3.88 ± 0.10	15.5	19.6

* On the 12th day, the deuteriomethionine content of the diet was increased from 0.5 to 1.0 per cent, as noted in the text.

† A is the deuterium content of the methyl group of the ingested methionine, 79.2 atom per cent deuterium. Since the deuteriomethionine of the diet is diluted by methionine in the fibrin of the diet, the values $B/A \times 100$ represent the *minimum* percentage of methyl groups of creatinine derived from dietary methionine.

TABLE II

Anserine, Choline, and Creatine Isolated from Rabbit Fed Deuteriomethionine for 28 Days

Compound isolated	Deuterium in isolated compound	Deuterium in methyl groups (B)	B/A* × 100
	atom per cent	*atom per cent*	
Anserine as copper anserine	0.28 ± 0.02	1.5	1.9
"Bound" choline as chloroplatinate	2.51 ± 0.06	3.9	4.9
"Free" " " "	4.68 ± 0.07	7.3	9.2
Creatine as creatinine K picrate	4.23 ± 0.09	16.9	21.4

* A is the deuterium content of the methyl group of the ingested methionine, 79.2 atom per cent deuterium.

of 70 per cent alcohol. An excess of Hopkins' reagent was indicated by the formation of a yellow precipitate instead of a white one. The precipitate, which at first was oily, hardened after standing overnight in the refrigerator. The mercury salt was washed with 75 per cent alcohol, then suspended in water, and decomposed with hydrogen sulfide. The mercury-free filtrate was neutralized with barium hydroxide. After removal of the barium sulfate the copper anserine was formed by the addition of an excess

of copper carbonate (about 2 gm. of $CuCO_3 \cdot 5H_2O$). The resulting solution was evaporated to dryness, the copper anserine was freed from inorganic material by extraction with absolute methyl alcohol, and the alcoholic solution was concentrated to 10 cc. Crystallization was effected by addition of 15 to 20 cc. of water, followed by removal of excess methanol by evaporation *in vacuo*. For complete precipitation of the crystals it was necessary to allow the solution to stand overnight in the refrigerator. The copper anserine was recrystallized twice by solution in methyl alcohol and addition of water as described above; 1.1 gm. of copper anserine, $C_{10}H_{16}N_4O_3CuO$, were obtained. The deuterium content of the copper anserine, as shown in Table II, was 0.28 ± 0.02 atom per cent, which corresponds to 1.5 atom per cent deuterium in the methyl group.

$C_{10}H_{16}N_4O_3CuO$. Calculated, N 17.5, Cu 19.8; found, N 18.0, Cu 19.5

Isolation of Choline—The liver, kidneys, and depot fat were frozen in solid CO_2 and ground in a fine meat chopper. The ground material was combined with the residue from the muscle tissue previously extracted for anserine. Choline was isolated from these sources as previously described (1). However, two separate fractions were isolated; namely the choline in the ether-soluble fraction, *i.e.* "bound" choline, and the choline in the water-soluble fraction, *i.e.* "free" choline. The so called "free" choline was actually a mixture of the various water-soluble choline derivatives plus some phospholipid choline liberated during the boiling alcohol extraction of the combined liver, kidneys, fat, and muscle tissue residue. 650 mg. of choline chloroplatinate were obtained from the "bound" choline fraction.

$C_{10}H_{28}N_2O_2PtCl_6$. Calculated, Pt 31.6; found, Pt 31.6

230 mg. of choline chloroplatinate were obtained from the "free" choline fraction.

$C_{10}H_{28}N_2O_2PtCl_6$. Calculated, Pt 31.6; found, Pt 31.5

The deuterium content of the "bound" and "free" choline differed considerably, as shown in Table II.

Isolation of Creatine from Tissues and Creatinine from Urine—The creatine was isolated in the manner previously described (1) from the filtrate obtained in the reineckate precipitation of the "free" choline fraction. 534 mg. of creatinine potassium picrate were isolated. Three samples of creatinine were isolated by the method already given (1) from urine samples which had been collected over 48 hour periods on the 7th and 8th, the 17th and 18th, and the 25th and 26th days respectively. As can be seen from a comparison of Tables I and II, the deuterium content of the creatine isolated from the tissue at the end of the 28 day experimental period agreed very well with the deuterium content of the urinary creatinine of the 25th and 26th days.

SUMMARY

The process of transmethylation occurs in the rabbit, as demonstrated by the appearance of deuterium in the choline and creatine of the tissues and the creatinine of the urine after the feeding of deuteriomethionine. The presence of deuterium in the anserine isolated from muscle tissue after the rabbit had been fed deuteriomethionine is consistent with the hypothesis that the various N-methyl compounds synthesized by the animal derive their methyl groups from "labile" methyl compounds of the diet.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

BIBLIOGRAPHY

1. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).
2. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **128**, p. cviii (1939).
3. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, **14**, 273 (1937).
4. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135**, 223 (1940).
5. Deutsch, A., Eggleton, M. G., and Eggleton, P., *Biochem. J.*, **32**, 203 (1938).
6. Hopkins, F. G., and Cole, S. W., *J. Physiol.*, **27**, 418 (1901–02).

BOUND PYRIDOXINE (VITAMIN B_6) IN BIOLOGICAL MATERIALS

By LOUIS SIEGEL, DANIEL MELNICK, and BERNARD L. OSER

(From the Food Research Laboratories, Inc., New York)

(Received for publication, May 21, 1943)

The method of Williams, Eakin, and McMahan (1) for the microbiological assay of pyridoxine is based upon the turbidimetric measurement of the growth response of the yeast *Saccharomyces cerevisiae*, Gebrüder Mayer strain.

Initial attempts to use the method in our laboratory were unsuccessful. The tests were complicated by excessive growth of the microorganism in all tubes, completely masking any gradation. This was found to be due to the pyridoxine remaining in the yeast and liver supplements despite treatment of these solutions with fullers' earth. The use of Lloyd's reagent yielded solutions free from pyridoxine. However, with the removal of the pyridoxine other growth factors essential for the microorganism were also adsorbed, necessitating their replacement to make the medium satisfactory.

Present Modifications of Microbiological Procedure

To the acidified (pH 1.0) liver and yeast extract solutions, described by the original authors (1), are added 2 gm. of Lloyd's reagent[1] and the suspension is shaken occasionally during the next 24 hours. After filtration the solution is treated again with the same quantity of the adsorbent. The mixture is then centrifuged, and the clear supernatant removed, adjusted to pH 4.5 to 5.0, bottled, and sterilized in the autoclave for 15 minutes at 15 pounds pressure.

To compensate for other growth factors adsorbed on the Lloyd's reagent along with the pyridoxine, it was necessary to increase the biotin[2] content of the medium to 0.1 γ and the casein hydrolysate[3] to 2.5 cc. per 100 cc. of medium. (The addition of other members of the vitamin B complex, riboflavin, choline, and nicotinic acid, failed to influence the growth of the microorganism.)

Tryptophane was found to be an essential growth factor and, therefore, is added in freshly prepared solutions, 0.2 mg. per 100 cc. of the basal medium.

[1] Obtained from Eli Lilly and Company, Indianapolis.

[2] For these tests, evaluating the importance of biotin in the medium, samples of crystalline biotin, kindly furnished by Dr. du Vigneaud of Cornell University Medical College, were used.

[3] Obtained from the S. M. A. Corporation, Chagrin Falls, Ohio.

The minerals and asparagine are added from a sterile stock solution containing 10 times the concentration required in the basal medium (1). The casein hydrolysate and vitamin solutions are stored separately, the latter in the refrigerator. The sucrose is dissolved as required.

The standard pyridoxine solution, 0.04 γ per cc., is prepared fresh daily from a stock solution. The latter is an acidulated aqueous solution (pH 3), containing 100 γ of pyridoxine per cc. It is stored in an amber bottle in the refrigerator. Since pyridoxine in other than acid solution is readily destroyed by visible light (2), all manipulations with this vitamin during the assay are conducted with minimal exposure to light.

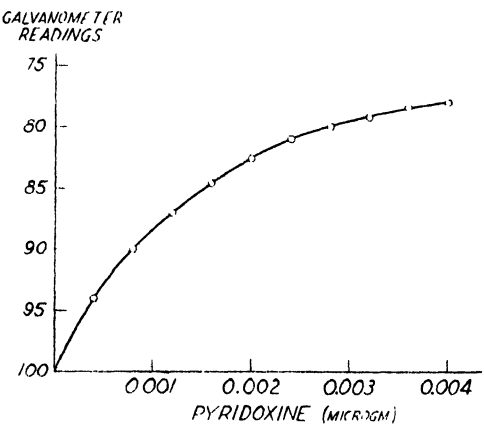


FIG. 1. Growth response of the yeast *Saccharomyces cerevisiae*, Gebrüder Mayer strain, to graded amounts of pyridoxine.

Acid hydrolysis of the aqueous suspension or solution of the test material is conducted in order to liberate bound pyridoxine. The importance of this modification is discussed in a later section of this report.

The culture is carried on Difco malt-agar slants at 30–31° and transplanted daily. At the end of 1 month the culture is discarded and a new series of daily transplants started from a stock culture which had been kept in the refrigerator for a period of 1 month. A new stock culture is also prepared at this time. A platinum loopful of the 24 hour surface growth is suspended in 20 cc. of sterile saline, drawn into a syringe, and each tube of medium inoculated by the drop method of Black and Arnold (3).

Rather than set up the standard solution at three assay levels in triplicate, we prefer to employ ten single assay levels varying at an arithmetical rate, as indicated in Fig. 1. The test solution or suspension is also tested at ten assay levels, but each approximately 25 per cent greater than the

preceding one, the volumes added to the 2 cc. quantities of medium varying from 0.01 to 0.1 cc. The tubes are plugged and the contents steam-sterilized for 10 minutes.

The yeast growth in the tubes incubated in a water bath at 31° is inhibited by the addition of chlorothymol at the end of 16 to 18 hours according to the published procedure (1). The photoelectric colorimeter is set to give a galvanometer reading of 100 with the blank tube[4] (inoculated but containing no pyridoxine) and the turbidities of the other tubes evaluated.[5] In the majority of cases color and turbidity in the test solutions introduce no significant error, since the volumes of the highly diluted test solutions added to the basal medium are small, 0.1 cc. or less. With colored or turbid solutions of very low potency, a blank correction may be necessary. Two uninoculated tubes, one containing 0.01 cc. and the other 0.1 cc. of the test solution, are read. The correction for interfering colors or turbidities of intermediate tubes may be interpolated from the linear graph for these tubes.

Hydrolysis of Bound Pyridoxine in Biological Materials—The method of Williams, Eakin, and McMahan (1) makes no provision for the hydrolysis of bound pyridoxine. That they measure only free pyridoxine is evident from the fact that their figures for pyridoxine content are much less than the values obtained by animal assays.

In Table I are presented the results of assays conducted on one of several rice bran concentrates. This particular product is of interest, since it had been carefully assayed independently in two other laboratories by two unrelated procedures, chemical (5) and biological (4). The microbiological assays were conducted against reference curves evolved with the standard pyridoxine solutions treated in the same manner as the test solutions. The rice bran concentrates, diluted 1:200 with sulfuric acid of indicated normalities, were autoclaved at 15 pounds pressure for 30 minutes, the pH

[4] A small amount of turbidity is always observed in the blank tube. This is not attributed to the presence of minimal quantities of pyridoxine in the medium, since prolonged irradiation (2) of those solutions (the liver, yeast, and amino acid supplements) which might possibly still contain some pyridoxine failed to lower significantly the blank value. Apparently the test organism can grow to a very limited extent, despite the absence of pyridoxine in the medium.

[5] An Evelyn photoelectric colorimeter, adapted for a 6 cc. aperture, may be used. The instrument is manufactured by the Rubicon Company, Philadelphia. In place of light absorption measurements, nephelometric readings may be obtained by using any of the standard fluorometers. The fluorometer is first set to read 100.0 with the suspension in the reference series showing maximal turbidity. It is possible to extend the turbidity range by increasing markedly the quantity of pyridoxine added to the reference tubes. However, better agreement among the various assay levels is consistently obtained when the tests are confined to the range indicated in the present paper.

adjusted to approximately 6.0 with 4 N sodium hydroxide solution, and then diluted to the testing range. It will be noted that heating the aqueous solutions for 10 minutes at 100° yields a value one-tenth of that obtained by biological assay. When the sample is subjected to acid hydrolysis in the autoclave, there is a marked progressive increase in the pyridoxine figures until a maximal value is obtained which closely approximates that yielded by the chemical and biological assays. Autoclaving in a more concentrated acid solution yields somewhat smaller pyridoxine values. An evaluation of the various reference curves evolved with the standard pyridoxine solution similarly treated indicates that no loss of the vitamin

TABLE I

Determination of Optimal Concentration of Acid for Hydrolysis of Bound Pyridoxine in Rice Bran Concentrates

Experiment	Pyridoxine found*
	γ per gm.
Simple solution in water, heated 10 min. at 100°	11
Acid hydrolysis + autoclaving at 15 lbs. pressure for 30 min.	
0.5 N H_2SO_4	56
1.0 " "	85
2.0 " "	115
3.0 " "	104
4.0 " "	100
5.0 " "	97

Other samples gave similar values when tested according to the above procedures. These were kindly furnished by the National Oil Products Company, Harrison, New Jersey.

* This same sample was found by Dr. Morgareidge of the above company to contain 110 γ per gm. according to the method of Dimick and Schreffler (4) in which both growth response and the degree of cure of the rat dermatitis are taken into consideration. Dr. Scudi of the Merck Institute for Therapeutic Research, Rahway, New Jersey, obtained 103 γ by his chemical procedure only after strong acid hydrolysis of the test solution (5).

occurred in the sulfuric acid solutions of 2 N or less and that only very slight destruction occurred in the more concentrated acid solutions. Accordingly, autoclaving the sample in solution or suspension for 30 minutes at 15 pounds pressure in 2 N sulfuric acid was selected as the most desirable procedure for hydrolyzing bound pyridoxine.

Atkin and collaborators (6) have also reported the necessity for hydrolyzing bound pyridoxine before reliable figures for total vitamin content can be obtained. For routine use they recommended autoclaving at 20 pounds pressure for 60 minutes in 0.055 N sulfuric acid solution. However, they recognized that this procedure was not universally applicable to all foods,

since they found that in the case of cereal products such as whole wheat and white flour it was necessary to increase the normality of the acid to 0.44.

TABLE II

Free and Bound Pyridoxine in Biological Materials

Sample	Pyridoxine content			Bound pyridoxine	Values reported in literature§
	Total*	Free†	Bound‡		
	γ per gm.	*γ per gm*	*γ per gm.*	*per cent*	*γ per gm.*
Whole wheat flour	4.2	0.9	3.3	79	4.8 (6), 4.6 (7)
Patent flour	0.9	0.4	0.5	56	1.2 (6), 2.2 (7)
Wheat germ	10.6	1.1	9.5	90	9.6 (7)
" bran	15.7	10.0	5.7	36	
Defatted soy bean flour	12.8	1.8	11.0	86	12 for ground soy beans (8)
" cottonseed meal	13.1	6.2	6.9	53	
Corn-meal	3.8	2.1	1.7	45	
Rice bran concentrate‖	115.0	11.0	104.0	90	100–140 (5), 137 (8)
Beef muscle	4.3	2.1	2.2	51	3.8–4.0 (9)
" liver	4.2	1.3	2.9	69	7.1 (6), 7.3 (7)
Pork loin	4.2	1.4	2.8	67	6.8 (6), 4.5–6.5 (9)
Fillet of flounder	1.0	0.3	0.7	70	
Whole milk	0.51	0.44	0.07	14	0 5–0.6 (6), 1 3 (9)
" hen's egg	0.48	0.39	0.09	19	
Dried whole "	2.3	1.8	0.5	22	
Liver extract powder	24.8	10 5	14.3	58	
Dried brewers' yeast	49.0	17.8	31.2	64	39 (6), 65–75 (8), 55 (10)
Yeast extract powder	90.0	34.0	56.0	62	
Blackstrap molasses	24.9	5.3	19.6	79	
Brown sugar	0.7	0.3	0.4	57	
	γ per sample	*γ per sample*	*γ per sample*		
24 hr. urine sample (L. S.)	65	13	52	80	127–143 (6)
24 " " " (D. M.)	172	35	137	80	

* Obtained on the acid-hydrolyzed suspension or solution after 30 minutes autoclaving of the sample at 15 pounds pressure in 2 N sulfuric acid.

† Obtained on the aqueous suspension or solution after heating for 10 minutes at 100°.

‡ Calculated by difference between total and free pyridoxine.

§ Values on similar samples reported in the literature by only the "more reliable" assay procedures are listed; the figures in parentheses refer to bibliographic citations.

‖ Represents an approximately 20-fold concentration of rice bran.

No rice bran preparations were included in their series of analyses. Our procedure involves hydrolysis in a much stronger acid solution but for a shorter period of time and at a lower pressure. The use of the strong acid

solution recommended does not destroy any pyridoxine and is in such excess as not to be affected appreciably by the buffering capacity of the test solution, regardless of the nature of the material being analyzed.

In Table II is presented a series of analyses conducted upon a variety of biological materials before and after acid autoclaving of the suspension or solutions yielding values for free and total pyridoxine. The bound pyridoxine is calculated by difference. It will be noted that in every case a large fraction of the pyridoxine is not readily available to the microorganism for growth. A comparison of the values for free and total pyri-

TABLE III

Reliability of Values for Total Pyridoxine

The values were obtained on the acid-hydrolyzed suspension or solution after 30 minutes autoclaving of the sample at 15 pounds pressure in 2 N sulfuric acid.

Sample	Microbiological assay values	Biological assay values*
	γ per gm.	*γ per gm.*
Dried liver Preparation A	6	6†
" " " B	16	20†
" " " C	25	30†
Rice bran Concentrate A	115	110‡
" " " B	100	100‡
Dried brewers' yeast	49	50
" yeast extract	90	85
B complex preparation	356	350
Multivitamin tablet	239	250

* The assays, unless indicated otherwise, were conducted in our laboratory by the method of Dimick and Schreffler (4) in which both growth response and the degree of cure of the rat dermatitis are taken into consideration.

† Assays reported by The Wilson Laboratories, Chicago, based on the rat growth method of Conger and Elvehjem (10).

‡ Assays conducted in the laboratories of the National Oil Products Company, Harrison, New Jersey, by the method of Dimick and Schreffler (4).

doxine with those reported in the literature by the "more reliable" assay procedures, which include the recent microbiological method of Atkin and associates (6) and the chemical method of Bina and collaborators (8), indicates that fairly good agreement is obtained only when the total pyridoxine values are considered. The figures for free pyridoxine cannot be regarded as constituting even a rough approximation of the vitamin B_6 potency.

Nine test samples, representing the four major sources of pyridoxine, were analyzed by both microbiological and biological methods of assay. The latter were conducted independently either in this laboratory or elsewhere, as indicated in Table III. The four types of test materials were

three natural vitamin B complex preparations, prepared from liver, rice bran, and brewers' yeast, and multivitamin preparations containing synthetic pyridoxine. The results indicate that in general good agreement between biological and microbiological assay on the same test sample may be expected, provided that in the latter case the test suspension or solution is first subjected to strong acid hydrolysis. Furthermore, assays by the microbiological procedure on a large number of freshly prepared pharmaceutical products, containing known quantities of pyridoxine in various admixtures with other vitamins and minerals, have yielded values in good agreement with the expected figures.

SUMMARY

1. A modification of the yeast growth method of Williams, Eakin, and McMahan for the determination of pyridoxine is described which eliminates many of the inadequacies of the original method.

2. A major portion of biologically active pyridoxine occurs in nature in the bound state.

3. Autoclaving the test suspension or solution for 30 minutes at 15 pounds pressure in 2 N sulfuric acid renders the bound pyridoxine available to the yeast cell, so that the results by the microbiological procedure agree with those obtained by biological assays.

4. The concentrations of free and total pyridoxine in a variety of biological materials are listed.

BIBLIOGRAPHY

1. Williams, R. J., Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub., No. 4137*, 24 (1941).
2. Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.*, **148**, 253 (1943).
3. Black, T. L., and Arnold, A., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 344 (1940).
4. Dimick, M. K., and Schreffler, C. B., *J. Nutrition*, **17**, 23 (1939).
5. Scudi, J. V., *J. Biol. Chem.*, **145**, 637 (1942).
6. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
7. Teply, L. J., Strong, F. M., and Elvehjem, C. A., *J. Nutrition*, **24**, 167 (1942).
8. Bina, A. F., Thomas, J. M., and Brown, E. B., *J. Biol. Chem.*, **148**, 111 (1943).
9. Waisman, H. A., and Elvehjem, C. A., The vitamin content of meat, Minneapolis (1941).
10. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).

INTESTINAL PHOSPHATASE*

By GERHARD SCHMIDT and S. J. THANNHAUSER

(From the Boston Dispensary, the Joseph H. Pratt Diagnostic Hospital, and Tufts College Medical School, Boston)

(Received for publication, April 28, 1943)

The recent discoveries of highly specific phosphatases such as diphosphoesterase, 5-nucleotidase, pyrophosphatase, and ribonuclease render it desirable to reinvestigate the specificity of alkaline phosphatase, since many properties of this enzyme have been studied on relatively crude and weakly active samples. Observations concerning the specificity of phosphatase are not only of interest in view of its biological rôle, but they also have an important bearing on the increasing use of this enzyme as a chemical tool for the structural elucidation of organic phosphorus compounds.

In this paper we will describe a method for the purification of alkaline phosphatase on a large scale. In addition we will report data concerning the specificity of the enzyme and its behavior toward activating and inhibiting substances.

The mucosa of the small intestines was selected as the source of the enzyme because of its very high content of alkaline phosphatase. Armstrong (1) succeeded several years ago in obtaining from dog feces very potent solutions of alkaline phosphatase. There is no doubt that the enzyme of his preparation originated from the intestinal mucosa. However, it seems to be preferable to prepare the enzyme from the mucosa proper, thus avoiding its exposure to intense bacterial action.

Methods

Phosphate was determined according to Fiske and Subbarow (2), Delory (3), or King and Delory (4). The latter methods were used whenever preliminary isolation of phosphate was desirable in order to avoid the interference of other substances with the colorimetric phosphate determination (fluoride ions, cysteine, nucleotides, protein split-products, pyrophosphate).

Determination of Phosphate in Presence of Pyrophosphate.—The presence of pyrophosphate in 0.03 mM concentration seriously interferes with all methods for the determination of inorganic phosphate. It prevents the precipitation of phosphomolybdate and the formation of molybdenum blue. Attempts to achieve a separation of phosphoric and pyrophosphoric acids

* This study was aided by grants from the Rockefeller Foundation, the Godfrey H. Hyams Trust Fund, the Bingham Associates Fund, and the Charlton Fund.

on the basis of the different solubilities of their barium salts had unsatisfactory results. We found, however, that the method of King and Delory (4) can be successfully applied to the determination of small amounts of phosphate in the presence of relatively large quantities of pyrophosphate. The high sensitivity of this method permits the use of such small aliquots for the analyses that the influence of the pyrophosphate becomes negligible. The original directions of King and Delory were modified in some details in order to adapt the method to phosphate-pyrophosphate mixtures. Since the precipitation of the phosphate as the hydroxyquinoline-molybdate complex is carried out in N hydrochloric acid, the heating of the mixture as recommended by King and Delory was omitted and the precipitate was allowed to stand for only 5 minutes at room temperature before centrifuging. We found it sufficient to scratch the wall of the centrifuge tube a few times with a glass rod in order to obtain the hydroxyquinoline-phosphomolybdate precipitate in a flocculent form. It was found by means of recovery experiments that amounts of phosphate above 2 γ can be quantitatively determined in aliquots containing not more than 100 γ of pyrophosphate phosphorus. In enzyme experiments with pyrophosphate it is necessary to determine the phosphate content of the pyrophosphate solution in a blank of a sample treated similarly to that incubated with the enzyme.

Substrates—Potassium diphenyl phosphate was synthesized according to the procedure of Asakawa (5). It was recrystallized several times from water in order to remove the last traces of the monoester.

Phosphopyruvic acid was synthesized from pyruvic acid and phosphorus oxychloride according to Kiessling (6). It was necessary, however, to modify the procedure of this author in some details, since we failed in several attempts to obtain the substance according to the original directions of Kiessling. The following modifications have been found to be useful.

1. Since the amount of quinoline used by Kiessling as a medium for the phosphorylation does not suffice to protect the phosphorylated product against the acid formed during the reaction, we took precautions preventing any increase of the acidity beyond the turning point of Congo red paper. The amount of quinoline present at the beginning of the phosphorylation was doubled in comparison to Kiessling's directions by dissolving the pyruvic acid in 3 times its weight of quinoline. During the course of the phosphorylation the acidity was frequently checked with the aid of Congo red paper and more quinoline added if necessary.

2. The preliminary fractionation of the crude barium salt, suggested by Kiessling, was omitted because we found that it had little purifying effect and caused great losses. We prepared the silver barium phosphopyruvate directly from the crude mixture of the barium salts and usually obtained

it immediately in well crystallized form. The salt never failed to crystallize after the first reprecipitation. Our yields amounted to 3 to 4 gm. of the silver barium salt from 40 gm. of pyruvic acid.

Determination of Phosphatase—Our method for the determination of phosphatase represents an adaptation of the existing procedures to the high activity of our enzyme preparations. It is based on the empirical fact that, during the initial stages of the hydrolysis of sodium β-glycerophosphate, the amounts of phosphate formed within a certain time interval are proportional to the amounts of phosphatase present.[1]

Standard Conditions—10 cc. of a 0.1 N veronal buffer of pH 9.30, 1 cc. of a 5 per cent solution of sodium β-glycerophosphate (Eastman), and 0.5 cc. of a 15 per cent solution of crystallized magnesium chloride are mixed in a test-tube and warmed to 37°. After addition of 1 cc. of the enzyme solution and incubation of the mixture for 15 minutes at 37°, the amount of inorganic phosphate was determined. If the amount of inorganic phosphorus exceeded 0.25 mg. (corresponding to 5 per cent hydrolysis), the determination was repeated with a conveniently diluted solution of the enzyme.

The unit of phosphatase is defined as the amount of enzyme required for the formation of 0.1 mg. of inorganic phosphorus within 15 minutes under the standard conditions. (Our standard conditions are suboptimal in regard to the pH and to the substrate concentration.)

Purification of Alkaline Phosphatase

The purification of the enzyme is based on its resistance to trypsin digestion, its low adsorption affinity for aluminum hydroxide, and on fractional precipitation by ammonium sulfate.

Extraction—Twenty pieces of calf intestines (the first 2 yards from the pylorus) are collected in the slaughter-house and thoroughly rinsed with tap water. No special precautions such as rapid preparation or cooling are required. The mucosa is squeezed out from each loop with the aid of wooden spatulas (tongue depressors). The material (usually 2 liters) is then diluted with 3 volumes of tap water and brought to a pH of 9.0 by slow addition of 2 N sodium hydroxide. After the addition of 500 cc. of toluene, the suspension is vigorously stirred for 30 minutes by a mechanical stirrer. The resulting milky suspension is strained through cheese-cloth (Yankee polishing cloth), leaving a very small mucus residue. The activity of such suspensions usually amounts to 30 to 50 units, the total nitrogen content to 2.5 to 3 mg. per cc.

Digestion with Trypsin—The suspension contains the phosphatase in

[1] Our studies on the kinetics of phosphatase have led us to the conclusion that the use of phenyl phosphate as substrate is preferable to that of glycerophosphate for the purpose of phosphatase determinations.

insoluble form associated with lipid particles, which cannot be separated by centrifugation from the liquid phase. The insolubility of the phosphatase can be demonstrated by filtration of the suspension through infusorial earth (Hyflo from Johns-Manville). As much as 85 per cent of the enzyme remains on the filter and can be recovered by simple water extraction of the filter cake.

Digestion with trypsin was found to be the only satisfactory way to render the enzyme soluble. It will be remembered that in most of the current procedures for the preparation of phosphatase, autolysis is used as an indispensable step for the liberation of the enzyme from the tissues (7). Artificial proteolysis in the form of trypsin digestion has been introduced as a useful step in the purification of alkaline kidney phosphatase by Ehrensvärd (8), who discovered that the activity of alkaline phosphatase is not affected by trypsin. Kutscher and Pany (9) found later that acid phosphatase is papain-resistant. A solution of 10 gm. of commercial trypsin (Difco) in 500 cc. of water is added to the suspension and the mixture is incubated for 24 to 36 hours at 37°. The digested material is mixed with 400 gm. of Hyflo Super-Cel and filtered on a large Buchner funnel over ordinary filter paper which had been covered with a layer of 400 gm. of Hyflo Super-Cel. The filtration proceeds rapidly and yields a clear yellow liquid. The filter cake is finally washed twice with 2 liters of water. Practically no loss of enzyme results from the tryptic digestion or from the filtration. The principal effect of the filtration is the removal of fats and fatty acids which are present in large amounts in the intestinal mucosa of young calves. At this stage the enzyme content per mg. of nitrogen of the solution amounts to 10 units.

Precipitation with 0.9 Saturated Ammonium Sulfate—The phosphatase is quantitatively precipitated from the combined filtrate and washings (total volume about 12 liters) by adding 600 gm. of solid ammonium sulfate, C.P., to each liter of the enzyme solution. After the solution has stood for a short while, the precipitate collects on top and can be easily separated from most of the mother liquor by siphoning. The top layer, containing the precipitate, is filtered over a thin layer of Hyflo Super-Cel on a Buchner funnel (diameter 25 cm.). The precipitate is suspended in about 300 cc. of 0.1 N ammonium acetate buffer of pH 8.9. (An approximately normal solution of this buffer is prepared in the following manner. 62 cc. of a 28 per cent solution of ammonia are diluted to 500 cc. After addition of 35 cc. of glacial acetic acid, the pH is adjusted to 8.9 by further dropwise addition of glacial acetic acid with thymol blue as indicator, and the volume is made up to 1000 cc.) The enzyme solution contains approximately 300 phosphatase units per mg. of nitrogen.

The solution of the ammonium sulfate precipitate is separated from in-

soluble contaminations by filtration over a thin layer of Hyflo Super-Cel. This solution is brownish and sometimes turbid and contains the total amount of phosphatase present in the original extract.

Fractionation with 0.8 Saturated Ammonium Sulfate—The amount of ammonium sulfate in the solution is approximately calculated from its specific weight, and enough solid ammonium sulfate is added to bring the ammonium sulfate content to 500 gm. for each 1000 gm. of water. The precipitate is filtered over a thin layer of Hyflo Super-Cel and washed with 200 cc. of 0.8 saturated ammonium sulfate solution.

This fractionation has only a relatively slight purifying effect, as judged from the activity per mg. of protein nitrogen in the precipitate which is only 1.5 times higher than in the solution of the first ammonium sulfate precipitate. It was found, however, that the step is indispensable for the success of the subsequent procedures. Apparently it removes contaminating proteins which cannot be separated from the phosphatase during the later stages of the purification.

Dialysis—The solution is prepared for the subsequent adsorption on aluminum hydroxide by dialyzing it overnight in collodion bags of 50 cc. capacity against 8 to 10 liters of 0.025 N ammonia-ammonium acetate buffer of pH 8.8. No loss of enzyme activity results from this procedure.

Treatment with Aluminum Hydroxide and Kaolin—The dialyzed solution of the second ammonium sulfate precipitate can be very efficiently purified by aluminum hydroxide, which adsorbs contaminating substances but leaves most of the enzyme in the solution. However, the selectivity of the adsorption holds true only as long as the adsorbent is not used in excessive amounts. *It is therefore important to add the adsorbent in small fractions and to control the activity in the supernatant after each addition.* The omission of this precaution can result in the loss of the whole preparation, since phosphatase, once adsorbed, cannot be recovered in soluble form.

10 cc. of a suspension of aluminum hydroxide Cγ (10) are added slowly with vigorous shaking to each 100 cc. of the dialyzed enzyme solution. After centrifugation, the activity per mg. of nitrogen is determined in the supernatant, and the adsorption of the supernatant is continued with smaller amounts (5 per cent) of aluminum hydroxide, until the enzyme activity per mg. of nitrogen reaches its maximum. At this stage the solution is only very slightly opalescent and almost colorless. One adsorption with 10 and three subsequent adsorptions with 5 per cent of the aluminum hydroxide suspension are usually sufficient to achieve the maximum purification possible. The amount of aluminum hydroxide used should be kept near the minimum required, since increasing amounts of phosphatase are lost in the solid phase as the purity of the enzyme approaches its maximum. If the adsorption is carried out cautiously, the loss does not

exceed 35 per cent of the amount of enzyme present before adsorption. The activity per mg. of nitrogen in the supernatant is usually 2400 units.

Removal of Contaminating Trypsin—All phosphatase preparations obtained by the procedure described above contain small amounts of trypsin. They give rise to definite increases in the formol titration and the amino nitrogen values when a solution containing 200 phosphatase units is incubated with 1 per cent gelatin at pH 8.5 for 24 hours. Such contaminations with trypsin would render impossible studies concerning the action of phosphatase on phosphoproteins.

The trypsin can be removed by shaking purified phosphatase solutions with 5 per cent kaolin and filtering the solution. When this procedure is repeated three times, no detectable amounts of trypsin are present in the resulting solution of phosphatase. Simultaneously the kaolin treatment practically eliminates small contaminations of ribonuclease. The loss of phosphatase incurred during the kaolin adsorption is negligible.

Precipitation of Phosphatase by Means of Acetone—At this stage of the preparation, the precipitation of phosphatase with ammonium sulfate has no further purifying effect. However, it can be used to obtain the enzyme in the form of a more concentrated solution. This is advisable if the concentration of phosphatase in the supernatant from the adsorption procedures is less than 200 units per cc. In this case the enzyme is precipitated with a yield of from 60 to 80 per cent by adding 60 gm. of ammonium sulfate to each 100 cc. of the supernatant. The precipitate is filtered over a thin layer of Hyflo Super-Cel, washed, and dissolved in a conveniently small volume of 0.05 N ammonia buffer. The ammonium sulfate is removed by dialysis as described before.

Usually, however, it is not necessary to concentrate the enzyme solution, and it is preferable to precipitate the supernatant directly with acetone. For this purpose 4 volumes of *redistilled, ice-cold* acetone are added slowly to 1 volume of the ice-cold supernatant. The mixture becomes opalescent when the concentration of acetone exceeds 45 volumes per cent, and a white, granular precipitate of microcrystalline appearance settles out after the mixture has stood for a short time in the refrigerator. The precipitate can be easily separated by centrifugation and is readily soluble in water. For reasons of stability it is dissolved in a small volume of 0.02 N ammonia buffer of pH 8.8. This solution contains 90 per cent of the phosphatase present in the supernatant from the adsorption. After one reprecipitation with acetone, the activity amounts to 4000 units per mg. of total nitrogen. The acetone precipitate can be dried *in vacuo* without loss of activity or solubility and represents the most suitable form of phosphatase for chemical analysis. However, we usually stored the enzyme as an aqueous solution which kept its activity unchanged for many months. The solution

of the second acetone precipitate was always dialyzed overnight in the refrigerator against 10 liters of 0.02 N ammonium acetate buffer of pH 8.9. Collodion bags were used for this purpose. The final yield of the enzyme amounts to approximately 30 per cent of that present in the intestinal mucosa. The outlined procedure was carried out on many different batches of material and gave easily reproducible results in regard to yield and purity of the enzyme.

Some Chemical Properties of Purified Phosphatase—All samples of purified phosphatase give positive biuret and Molisch tests. They reduce alkaline copper solutions after hydrolysis with N hydrochloric acid. The polysaccharide content of purified phosphatase is very considerable. We determined the carbohydrate content in several phosphatase preparations according to the method of Somogyi (11). In the following, we report the results of such an analysis.

5 cc. of a dialyzed phosphatase solution contained 8.2 mg. of dry material. The solution was free of ammonia and gave 2.2 mg. of ash after combustion in a platinum crucible in an electric furnace.

2 cc. of the solution contained 0.242 mg. of total nitrogen, according to the method of Kjeldahl.

0.01 cc. of the solution formed 0.485 mg. of inorganic phosphorus under standard conditions, corresponding to a content of 4000 phosphatase units per mg. of nitrogen.

A glucose determination according to Somogyi was carried out on 3 cc. of the solution. No glucose was found.

3 cc. of the solution were refluxed with N hydrochloric acid for 3 hours. After neutralization, the volume was made up to 10 cc. A glucose determination according to Somogyi was carried out in an aliquot of 3 cc. The solution consumed 2.1 cc. of a 0.005 N solution of iodine, corresponding to a carbohydrate content of 0.254 mg. (calculated as glucose).

From the reported values it was calculated that the analyzed sample of phosphatase contained 10.1 per cent nitrogen and 21.2 per cent polysaccharide (calculated as glucose).

It is obvious that the values per 100 gm. of dry weight represent only approximate estimations, since it is arbitrary to consider the total amount of ash as a contamination.

DISCUSSION

The presence of a polysaccharide in highly purified samples of phosphatase is of considerable interest in view of the readiness with which phosphate reacts with carbohydrates in the organism. On the other hand, it is well known how tenaciously polysaccharides can adhere to proteins during the procedure of purification. It will be possible to decide by elec-

trophoresis or sedimentation experiments whether the polysaccharide is a constituent of the phosphatase molecule or a contamination. Unfortunately, an examination of phosphatase by these methods could not be carried out in our laboratory owing to the lack of the special equipment.

Data Concerning Kinetics of Alkaline Phosphatase

Influence of Substrate Concentration on Activity of Alkaline Phosphatase—It is obvious that the description of phosphatase would be greatly facilitated if the effect of this enzyme on its various substrates could be characterized by the computation of Michaelis-Menten constants. The knowledge of these constants would enable us to compare the behavior of the enzyme towards various substrates under given temperature and pH conditions without reference to the substrate and enzyme concentrations in the individual experiments.

Unfortunately the application of the Michaelis-Menten equations to the action of alkaline phosphatase is at present very limited, owing to the peculiar kinetics of the action of this enzyme. When we investigated the action of phosphatase on three typical substrates, phenyl phosphate, β-glycerophosphate, and inorganic pyrophosphate, we found that only the hydrolysis of phenyl phosphate represents a unimolecular reaction. Only in this case was it possible to determine the initial rates of the enzyme action with sufficient accuracy to allow the application of the Michaelis-Menten equation. Fig. 1 represents an experiment with phenyl phosphate in which the influence of the substrate concentrations on the rate of hydrolysis was investigated (Curve A). The Michaelis-Menten constants as computed according to Lineweaver and Burk (12) were found to be 7.20×10^{-5}, 7.20×10^{-5}, 7.30×10^{-5}, 7.20×10^{-5} for substrate concentrations of 1.8, 0.9, 0.54, 0.36 mM respectively (Curve B). The amount of phosphatase present in all samples of this experiment was 0.19 unit. In another experiment in which 0.045 unit of enzyme was used the values for the constant were 7.8×10^{-5} and 7.5×10^{-5} for substrate concentrations of 0.54 and 0.36 mM respectively. The agreement between the results obtained with two different enzyme concentrations appears satisfactory in view of the circumstance that the low substrate concentrations at which the rate of hydrolysis approaches its maximum necessitates the use of very small amounts of enzyme in order to keep the extent of the hydrolysis below 5 per cent of the total amount of substrate. The values obtained with the larger amount of enzyme should be considered more reliable than those with the smaller amount of enzyme. It might be noted that the range of substrate concentrations at which the rate of hydrolysis reaches its maximum agrees well with that observed by Folley and Kay (13) for the alkaline phosphatase of the mammary gland. These authors found in addition an inhibitory

effect of substrate concentrations beyond the optimum and considered the possibility that the enzyme forms at least two compounds with the substrate. They assumed that only one of these compounds forms phenol and inorganic phosphate on decomposition (14). We have extended our investigation over a range of substrate concentrations from 0.8 mM up to 125 mM and found indeed a slight inhibitory effect in the higher ranges of concentrations (Fig. 1, Curve C, and Fig. 2). However, it seems to us that this effect is too small to warrant the application of Haldane's equation or to influence appreciably the calculation of the Michaelis-Menten constant,

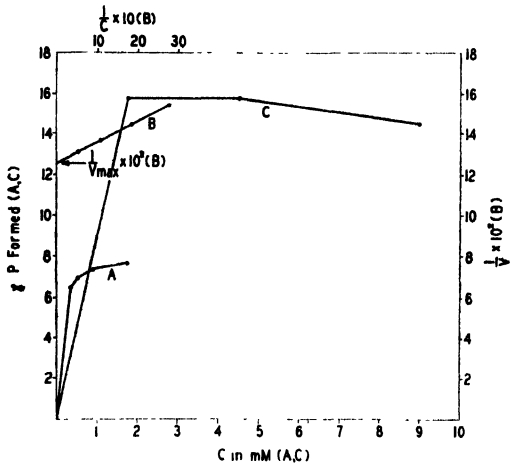


FIG. 1. Curve A, influence of the substrate concentration on the enzymatic hydrolysis of sodium phenyl phosphate. Curve B, curve obtained from the data of Curve A according to Lineweaver and Burk. (c, substrate concentration in mM; v, micrograms of phosphate formed during 4 minutes of incubation.) Amount of phosphatase, 0.19 unit; time of incubation, 4 minutes; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer of pH 9.23. Curve C, influence of the substrate concentration on the enzymatic hydrolysis of sodium phenyl phosphate. The amount of enzyme used was twice as much as that used for Curve A. The curve covers a higher range of substrate concentrations.

which is based on the rates of hydrolysis in the range between 0.36 and 1.8 mM substrate concentrations.

On hydrolysis, β-glycerophosphate behaves somewhat differently from phenyl phosphate, inasmuch as the enhancing influence of increasing substrate concentrations is observed up to the highest investigated concentrations without the existence of an asymptotic part of the curve (Fig. 3).

Pyrophosphate, which is split by phosphatase at low concentrations, progressively inhibits the action of the enzyme in the ranges of higher concentrations until the activity of the enzyme practically ceases (Fig. 4).

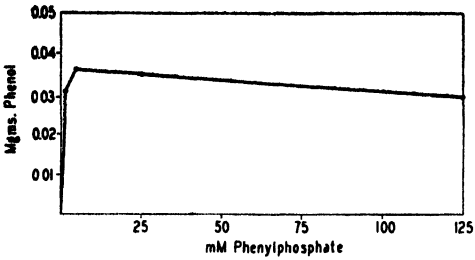


FIG. 2

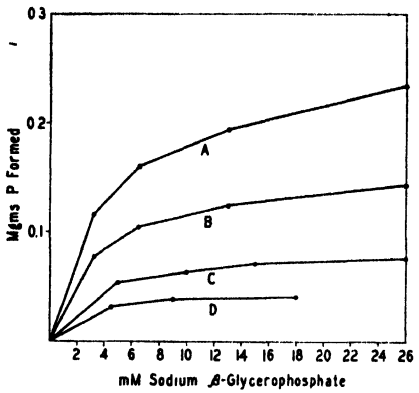


FIG. 3

FIG. 2. Influence of the substrate concentration on the enzymatic hydrolysis of sodium phenyl phosphate over a wide range of substrate concentrations. Amount of phosphatase, 0.25 unit; time of incubation, 5 minutes; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer of pH 9.23.

FIG. 3. Influence of the substrate concentration on the enzymatic hydrolysis of sodium β-glycerophosphate. Amount of phosphatase, 6, 3, 1.5, and 0.75 units in Curves A, B, C, and D respectively. Total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer of pH 8.9 Time of incubation, 5 minutes.

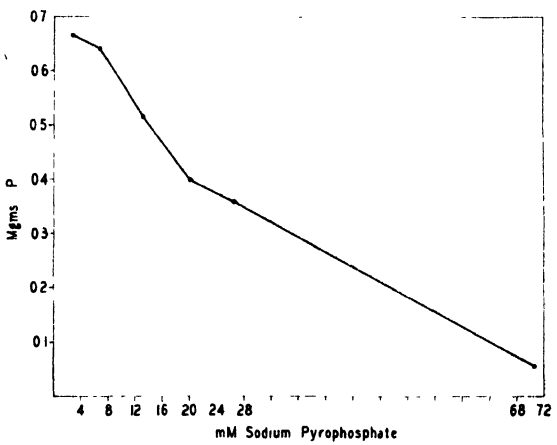


FIG. 4. Influence of the substrate concentration on the enzymatic hydrolysis of sodium pyrophosphate. Amount of enzyme, 28 units; time of incubation, 20 minutes; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer of pH 9.15.

The concentration at which the inhibition is complete depends on the amount of enzyme present (Table I). 28 units of phosphatase are completely inhibited in 80 mM pyrophosphate solution. The observations

concerning the pyrophosphate inhibition of phosphatase suggest the idea that phosphatase might be a metal protein capable of forming complexes with pyrophosphate.

TABLE I

Influence of Concentration of Phosphatase on Concentration of Pyrophosphate Necessary for Total Inhibition of Phosphatase Action

Time of incubation, 20 minutes; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer; concentration of pyrophosphate, 105 mM; pH 9.32.

Phosphatase	Phosphorus formed	Per cent hydrolysis
units	*mg.*	
18.5	0	0
185	0.66	0.9
925	3 57	4.85

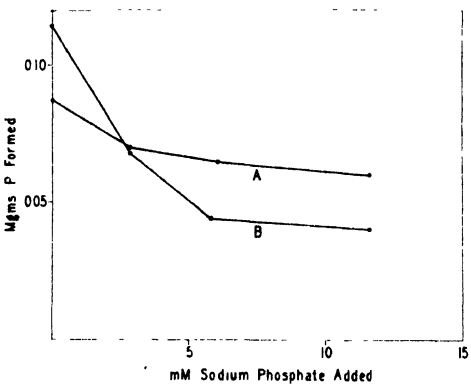


FIG. 5

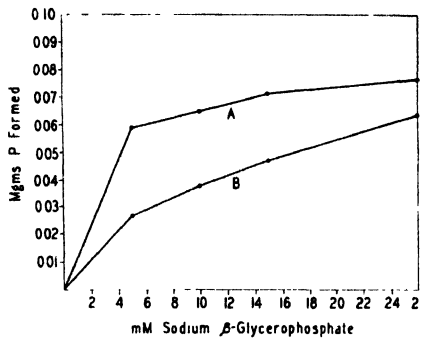


FIG. 6

FIG. 5. Influence of the addition of various amounts of sodium phosphate on the hydrolysis of sodium phenyl phosphate (Curve A) and sodium β-glycerophosphate (Curve B). Amount of phosphatase, 2.5 units; time of incubation, 5 minutes; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer; pH 9.15.

FIG. 6. Influence of sodium phosphate (1 mM) on the hydrolysis of sodium β-glycerophosphate in presence of varied amounts of substrate. Curve A, no sodium phosphate added; Curve B, 1 mM sodium phosphate added; amount of phosphatase, 1.7 units; time of incubation, 5 minutes; total volume, 12.5 cc containing 10 cc. of 0.1 N veronal buffer; pH 9.15.

Influence of Split-Products—While the alcohol moieties of the substrates are practically without influence on the rate of phosphatase action in the range of low concentrations, a very marked inhibitory effect of phosphate ions is characteristic for the hydrolysis of all investigated substrates (Fig. 5). The effect is at least partially competitive, since its intensity lessens with increasing substrate concentrations (Fig. 6). It is plausible to assume

that phosphatase combines with its substrate at the phosphate radical exclusively. This assumption would also explain the lack of specificity of phosphatase action.

Time Curves—Out of all investigated examples of phosphatase action, only the hydrolysis of phenyl phosphate follows the course of a unimolecular reaction. In the case of the other investigated substrates the rates drop faster (Figs. 7 and 8). Although the phosphate inhibition is in part responsi-

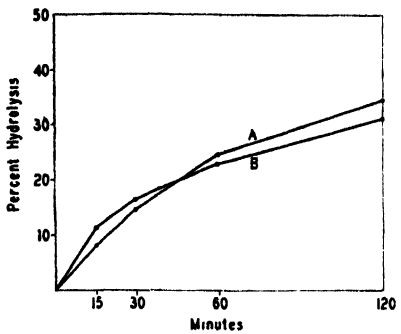


FIG. 7

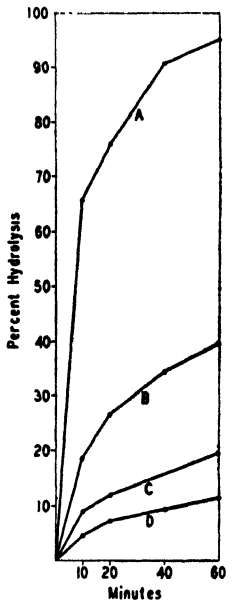


FIG. 8

FIG. 7. Time curves of the enzymatic hydrolysis of sodium monophenyl phosphate (Curve A) and sodium β-glycerophosphate (Curve B). Amount of phosphatase, 3.6 units; concentration of substrate, 8.5 mM; total volume, 11 cc. containing 10 cc. of 0.1 veronal buffer; pH 9.15.

FIG. 8. Time curves of the enzymatic hydrolysis of sodium pyrophosphate in presence of varied amounts of phosphatase. Amounts of enzyme, 120, 12, 4.8, and 2.4 phosphatase units in Curves A, B, C, and D respectively; concentration of substrate, 6.5 mM; total volume, 11 cc. containing 10 cc. of 0.1 N veronal buffer; pH 8.9.

ble for this behavior, it is not yet possible to decide whether it is the only reason for the peculiar slope of the time curves.

Activators and Inhibitors of Alkaline Phosphatase

Magnesium Ions—The influence of added magnesium salts on intestinal phosphatase is very slight (Table II). This fact has already been emphasized by Armstrong (1). Since the enhancing effect of magnesium salts

is still increasing in the ranges of rather high concentrations, it is difficult to decide whether the effect is due to a specific activation of the enzyme or simply to the removal of phosphate ions.

Amino Acids (Cysteine Excluded)—No appreciable effect of glycine or alanine on the action of phosphatase could be detected. Hove, Elvehjem, and Hart (15) reported in 1940 that zinc salts in combination with amino acids have an activating effect on dialyzed intestinal phosphatase from rats. In our experiments with dialyzed and purified phosphatase from calves, the addition of 1.5 mM alanine and 0.05 mM zinc sulfate, either

TABLE II

Influence of Magnesium Ions on Intestinal Phosphatase

Total volume, 11.5 cc. containing 10 cc. of 0.1 N veronal buffer of pH 9.2; 12.5 mM β-sodium glycerophosphate; 1.35 phosphatase units; incubation, 15 minutes at 37°.

Magnesium chloride, *mM*	0	0.26	1.3	6.5	32
Phosphorus formed, *mg.*	0.110	0.123	0.125	0.129	0.141

TABLE III

Inhibitory Effect of Cysteine on Intestinal Phosphatase

Standard conditions, 2.5 units of phosphatase.

Cysteine added, *mM*	0	0.5	1.0	2.0	5.0
Inorganic phosphorus formed, *mg.*	0.248	0.236	0.164	0.092	0.064

TABLE IV

Influence of $(CN)^-$ Ions

Total volume, 13 cc. containing 10 cc. of 0.1 N veronal buffer of pH 9.2; 12.5 mM β-glycerophosphate; 2.5 phosphatase units.

NaCN, *mM*	0	3.0	7.5	15.0
Phosphorus formed in 15 min., *mg.*	0.203	0.104	0.089	0.026

separately or in combination, had no influence on the activity of the enzyme.

Bile Acids—No effect on the activity of phosphatase was observed on addition of sodium cholate in various concentrations.

Fluorides—Fluorides up to 50 mM concentrations have no effect on intestinal alkaline phosphatase.

Cysteine—Several investigators have observed the strong inhibitory effect of cysteine on alkaline phosphatases prepared from various sources (16, 17, 18). We obtained similar results with purified intestinal phosphatase (Table III).

Cyanide—The data reported in Table IV demonstrate the strong in-

hibitory effect of cyanides in small concentrations. This result supports the view already suggested by the effect of pyrophosphate that phosphatase is probably a metal protein compound.

Specificity

Adenyl Pyrophosphate—Fig. 9 demonstrates the action of exhaustively dialyzed phosphatase on adenyl pyrophosphate. The course of the reaction was followed up to a point at which 78 per cent of the substrate was hydrolyzed. While it is evident that all three phosphate linkages are attacked by the enzyme, it appears that the two acid-hydrolyzable linkages are split faster than the non-hydrolyzable ester linkage. This observation is in complete agreement with the results obtained by Liebknecht (19) in Lohmann's laboratory with bone phosphatase. It is in contradiction to the observations of Barrenscheen (20) who claims that only the non-

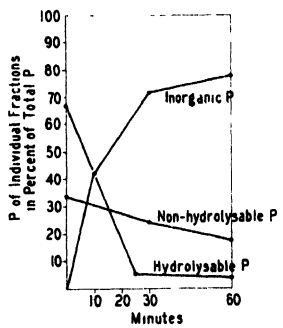


FIG. 9. Hydrolysis of adenyl pyrophosphate by alkaline phosphatase. Amount of phosphatase, 500 units; substrate concentration, 7 mM; total volume, 11 cc.

hydrolyzable phosphate linkage is split by alkaline phosphatase. We convinced ourselves that adenyl pyrophosphate is stable under the conditions of our experiment in the absence of phosphatase.

Phosphopyruvic acid is split by intestinal phosphatase. 40 units of the dialyzed enzyme are sufficient to release 85 per cent of the total phosphorus from 10 cc. of a 10 mM solution within 60 minutes.

Diphenylphosphoric Acid—King and Delory (21) reported in 1939 that several diesters of phosphoric acid are hydrolyzed by Armstrong's phosphatase. Our own observations agree with the results of these authors. We found that diphenyl phosphate is split by intestinal phosphatase at a rate which is 170 times lower than that of the hydrolysis of the monoester. The time curve represents practically a straight line up to 7 per cent hydrolysis. The split-products are phenol and inorganic phosphate which appear at a molecular ratio of 2:1 throughout the course of the hydrolysis.

In view of the much faster rate at which the monoester is split, this fact does not exclude the assumption of a stepwise degradation.

Very likely the hydrolysis of the diester is caused by the action of phosphatase itself and not by the presence of contaminating amounts of a hypothetical "phosphodiesterase." When we attempted to isolate the enzyme responsible for the hydrolysis of the diester from the intestinal mucosa, we found it in each stage of the preparation associated with the phosphatase-containing fractions. These observations are of course not in contradiction to the existence in snake venom of a specific phosphodiesterase, but it appears that the term "phosphomonoesterase," sometimes used in the literature for phosphatase, is misleading.

Biological Diesters of Phosphoric Acid—Cephalin, prepared according to Parnas (22), *lecithin*, prepared from egg yolk and brain according to Levene and Rolf (23), and *sphingomyelin*, prepared according to Thannhauser and Setz (24), are not attacked even by large amounts of intestinal phosphatase (700 units in 10 cc. of 8.5 mM emulsion of the substrates).

Yeast nucleic acid[2] is completely dephosphorylated in the presence of sufficiently large amounts of phosphatase. The absence of ribonuclease in the phosphatase solutions used for the hydrolysis was demonstrated by the complete resistance of ribonucleic acid to phosphatase solutions which had been heated at pH 5.0 to 80° during 15 minutes.

In view of the increasing use made of phosphatases in studies concerning the structure of nucleic acids (25, 26), we wish to emphasize that alkaline phosphatase offers no simple possibilities for the structural differentiation of the various phosphorus radicals in ribonucleic acid.

Thymonucleic acid in its highly polymerized form (prepared according to Hammarsten (27)) is not split by phosphatase.

It is interesting to note the different behavior of ribonucleic acid and desoxyribonucleic acid towards hydrolyzing enzymes. The nucleotides of ribonucleic acid, which can be so easily obtained by alkali hydrolysis of ribonucleic acid at room temperature, have never been observed as products of its enzymatic hydrolysis. The degradation by ribonuclease stops at split-products of higher molecular weight, while the hydrolysis by phosphatase leads to the complete release of the phosphorus radicals without the intermediary action of another enzyme.

On the other hand, it has been conclusively demonstrated by Thannhauser and his coworkers (28) that the enzymatic formation of nucleotides from desoxyribonucleic acid is the necessary intermediary reaction preceding the formation of nucleosides and inorganic phosphate.

Casein is not split by phosphatase, but is readily dephosphorylated by the

[2] The substance was obtained from Schwartz Laboratories, Inc., New York. It was purified by two precipitations with glacial acetic acid.

enzyme after previous treatment with commercial trypsin. This observation suggests the assumption that the phosphorus groups in casein are not present as "end-groups," but participate in the linkages between the amino acids.

It will be of interest to investigate which one of the proteolytic components of crude trypsin—trypsin, chymotrypsin, or carboxypolypeptidase—renders the phosphate radicals in casein susceptible to the action of phosphatase. Such information might be helpful in characterizing the different specificities of trypsin and chymotrypsin.

SUMMARY

1. A method for the purification of alkaline intestinal phosphatase is described.

2. The kinetics of the enzymatic hydrolysis of sodium phenyl phosphate and sodium β-glycerophosphate have been studied.

3. The influence of some substances on the activity of purified alkaline phosphatase has been investigated. It was found that the enzyme was inhibited by small amounts of cysteine and cyanide ions, but not by fluoride ions. Magnesium ions had a very slight activating effect. Zinc salts, alanine, and bile salts were without effect.

4. The specificity of alkaline intestinal phosphatase has been studied. Phosphopyruvic acid, pyrophosphoric acid, adenylpyrophosphoric acid, and diphenylphosphoric acid were hydrolyzed, while phosphatides were found to be completely resistant to the enzyme. The phosphoric acid radicals of yeast nucleic acid were completely released as inorganic phosphate in the presence of intestinal phosphatase, while highly polymerized thymonucleic acid was not split under the same conditions. Casein and phosphovitellin were not attacked by the enzyme, but it was found that the phosphoric acid radicals of these phosphoproteins were readily liberated as inorganic phosphate after preliminary treatment of the proteins with crude trypsin.

5. As much as 20 per cent of a polysaccharide was regularly found to be present in samples of purified phosphatase.

BIBLIOGRAPHY

1. Armstrong, A. R., *Biochem. J.*, **29**, 2020 (1935).
2. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
3. Delory, G. E., *Biochem. J.*, **32**, 1161 (1938).
4. King, E. J. and Delory, G. E., *Biochem. J.*, **31**, 2046 (1937).
5. Asakawa, K., *J. Biochem.*, Japan, **11**, 143 (1929).
6. Kiessling, W., *Ber. chem. Ges.*, **69 B**, 2331 (1936).
7. Albers, H., and Albers, E., *Z. physiol. Chem.*, **232**, 189 (1935).
8. Ehrensvärd, G., *Z. physiol. Chem.*, **217**, 274 (1933).
9. Kutscher, W., and Pany, J., *Z. physiol. Chem.*, **255**, 169 (1938).
10. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56**, 1117 (1923).

11. Somogyi, M., *J. Biol. Chem.*, **117**, 771 (1937).
12. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
13. Folley, S. J., and Kay, H. D., *Biochem. J.*, **29**, 1837 (1935).
14. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London and New York, 84 (1930).
15. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **134**, 425 (1940).
16. Waldschmidt-Leitz, E., Scharikova, A., and Schäffner, A., *Z. physiol. Chem.*, **214**, 75 (1933).
17. Kiese, M., and Hastings, A. B., *Science*, **88**, 242 (1938).
18. Sizer, I. W., *J. Biol. Chem.*, **145**, 405 (1942).
19. Liebknecht, W. L., *Biochem. Z.*, **303**, 96 (1939).
20. Barrenscheen, H. K., and Jachimowitz, T., *Biochem. Z.*, **292**, 350 (1937).
21. King, E. J., and Delory, G. E., *Biochem. J.*, **33**, 1185 (1939).
22. Parnas, I. K., *Biochem. Z.*, **56**, 17 (1913).
23. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **72**, 587 (1927).
24. Thannhauser, S. J., and Setz, P., *J. Biol. Chem.*, **116**, 527 (1936).
25. Gulland, J. M., and Jackson, E. M., *J. Chem. Soc.*, 1492 (1938).
26. Bolomey, R. A., and Allen, F. W., *J. Biol. Chem.*, **144**, 113 (1942).
27. Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).
28. Klein, W , and Thannhauser, S. J , *Z. physiol. Chem.*, **218**, 173 (1933)

A QUANTITATIVE METHOD FOR THE DETERMINATION OF TYROTHRICIN

By KEENE P. DIMICK

(From the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

(Received for publication, May 25, 1943)

Investigations in progress in this laboratory concerning the usefulness of vegetable juices as media for growing antibiotic-producing organisms required a rapid and accurate method for the quantitative determination of tyrothricin. The usual assay method for tyrothricin is based on the inhibitory effect of this substance on the growth of various test organisms such as *Lactobacillus casei*, *M. Y. coccus* (an unidentified micrococcus obtained from Merck and Company, Inc.), and *Staphylococcus aureus*. By the serial dilution technique it is possible to arrive at values roughly indicating the concentration of tyrothricin. As routinely carried out, this method is, however, subject to the usual limitations of such methods; namely, low degree of accuracy, slowness, and variations in the test organisms.

Another method, which probably gives more accurate values than the microbiological method, is the actual isolation of tyrothricin from the culture media by the method of Dubos and Hotchkiss (1). As will be shown later in this paper, about 75 per cent of the activity is usually recovered by this procedure.

The hemolytic properties of tyrothricin[1] have been reported by various workers (1–5). Apparently the possibility of utilizing this property for assay purposes has not been investigated. In planning the research reported here it was considered that, if this action is inherent in the bactericidal substance, it would be possible to measure the degree of hemolysis of red blood cells and to establish a relationship between the amount of bactericide and the degree of hemolysis. If this relationship could be established, a quantitative determination of tyrothricin in unknown solutions or media should be possible. Experimental work has substantiated this idea, and a method has been developed by means of which it is possible to determine as little as 100 mg. of tyrothricin per liter of media, with an accuracy of about 5 per cent.

[1] Although tyrothricin is a mixture of two substances (tyrocidine and gramicidin), the proportion of the two, so far as is known, is constant in culture media and in isolated material (2).

EXPERIMENTAL

Preparation of Standard Tyrothricin Solution—A stock solution is prepared by adding 50 mg. of tyrothricin to 1 liter of 95 per cent alcohol. The tyrothricin used in these experiments was isolated, by the method employed by Dubos and Hotchkiss (1) with a slight modification, from *Bacillus brevis* culture grown on media made from an extract of dehydrated asparagus butts. The activity of this standard solution, as determined by the method described in this communication, was found to be comparable, within experimental limits, to the activities of (1) a sample of tyrothricin furnished by Merck and Company, Inc., and (2) mixtures of crystalline tyrocidine hydrochloride and crystalline gramicidin in proportions ranging from 5 to 35 per cent of gramicidin.

Preparation of Stock Erythrocyte Suspension—Rats of a Sprague-Dawley strain were used as a source of the erythrocytes. The rats were anesthetized with chloroform, and the blood was drawn from the heart into a syringe containing 0.5 ml. of saturated sodium oxalate solution. The amount of blood (about 5 ml.) was measured to the nearest 0.1 ml., and after being mixed was diluted with 85 ml. of 0.87 per cent saline. The suspended cells were then centrifuged, and the supernatant was carefully poured off. The cells were then diluted with 0.87 per cent saline to 10 times the original blood volume, and were stored in an ice chest. The stock preparation must be freshly prepared every 3 or 4 days.

Analytical Procedure

Ponder has pointed out in a series of publications (6) that the degree of hemolysis of red blood cells can be accurately measured by colorimetric procedures. For the present investigation a Klett-Summerson photoelectric colorimeter, equipped with a 660 mμ filter, was employed. This filter allows a maximum of light to be transmitted with hemolyzed blood, and the maximum change in transmission between the unhemolyzed and the hemolyzed cells is obtained.

For test purposes the following method is used. 5 ml. of the stock blood preparation are diluted to 190 ml. with 0.87 per cent saline and placed in a 500 ml. Erlenmeyer flask. This enables the operator to shake the suspended cells before each sample is removed. After this diluted suspension has stood for about 15 minutes, it is shaken a time or two, and 10 ml. are pipetted into a calibrated colorimeter tube. The initial reading of this cell suspension should be between 80 and 90 divisions on a Klett-Summerson colorimeter which has been adjusted to zero with distilled water. This initial reading may fluctuate two or three divisions, but the average reading should be constant for several hours and should be used in the calculations.

A calibration curve must be prepared for each diluted blood preparation.

For this purpose seven standard tyrothricin solutions are made by diluting the stock solution with 95 per cent alcohol. These standards contain 8 to 20 γ of tyrothricin per ml. of alcohol.

The initial reading of the standard cell suspension is checked, and the tube is removed from the instrument. 0.5 ml. of one of the standard tyrothricin solutions is added with a 1 ml. serological pipette and, in order to mix the contents, the thumb is placed over the opening of the tube which is inverted slowly four times. The tube must not be shaken, since the mixing has been found to be somewhat critical, and the directions given should be

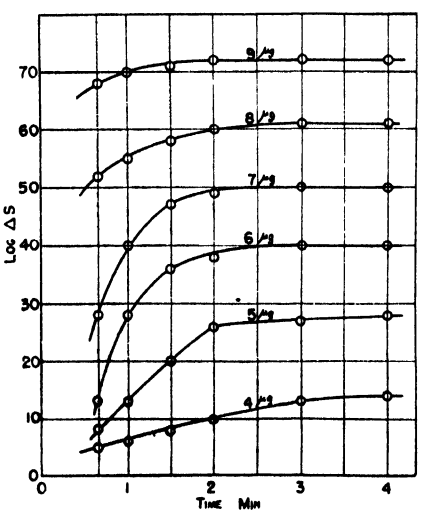


FIG. 1

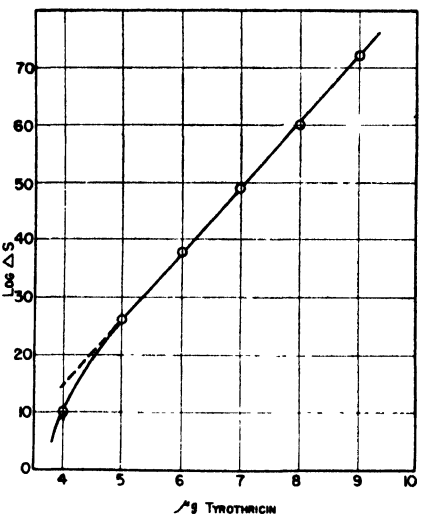


FIG. 2

FIG. 1. The rate of hemolysis of a standard erythrocyte suspension with varying amounts of added tyrothricin. ΔS = change in transmission of light.

FIG. 2. Calibration curve. The relationship between the micrograms of tyrothricin and hemolysis of a standard erythrocyte suspension in 2 minutes.

followed precisely. A stop-watch is started at the time of the fourth inversion, and readings are taken at 40, 60, 90, 120, and 180 seconds. These values are subtracted from the average initial reading and recorded. They represent the log of the change in transmission of light (ΔS). By plotting log ΔS against time for various concentrations of tyrothricin a family of curves is obtained (Fig. 1).

It was found necessary to add the tyrothricin dissolved in alcohol to the blood cells in order to get reproducible results. This may be due to the low solubility of tyrothricin in water. With alcohol as a solvent a simple quantitative extraction of the culture media is also made possible. Alco-

hol itself has little effect on the blood cells in concentrations up to 10 per cent in 10 or 15 minutes. It does show, however, a marked inhibitory effect on the hemolytic action of the tyrothricin. For this reason it is necessary to add a constant amount (0.5 ml. of alcohol to 10 ml. of the blood cells). If this procedure is followed, the inhibitory effect of the alcohol is constant.

In order to prepare the calibration curve (Fig. 2), the ΔS values are taken from the flat portion of each curve (the 2 minute reading), and these values are plotted against their respective tyrothricin concentrations. In this manner a straight line is obtained which will relate the tyrothricin concentration with the change in transmission. Thus, it becomes possible to determine the tyrothricin content of any given alcoholic solution by measuring the change in transmission which is caused by 0.5 ml. of this solution.

The calibration curves have been constructed from many different samples of blood obtained from different rats, and in all cases the slopes have

TABLE I

Dilution Factors for 1 Ml. of Alcoholic Extract for Hemolytic Assay Procedure

Alcohol added	Factor
ml.	
0	20
1	40
2	60
3	80
4	100

been similar. This has been found true also for different samples of the same stock of erythrocytes taken on different days.

Since the range of this test is rather narrow, covering generally 5 to 10 γ per 0.5 ml. of alcohol, it is necessary to adjust the concentration of an unknown alcohol solution obtained from the extraction of the culture medium so that its concentration will fall within these limits. This is done by diluting the alcohol solution with pure 95 per cent alcohol (see Table I).

The assay of dry isolated material may be accomplished by simply weighing out a sample and dissolving it in a definite volume of alcohol.

For the assay of culture media, 1.0 ml. of a thoroughly shaken culture is pipetted into a 15 ml. conical centrifuge tube. 9 ml. of 95 per cent alcohol are then added, and the tube is shaken vigorously. This shaking forms a fine suspension of cells in the alcohol. The suspended, extracted cells, after standing $\frac{1}{2}$ hour, are centrifuged off. Dilutions of this alcoholic extract with alcohol are made, and the resulting solution is assayed as previously described.

The number of micrograms of tyrothricin per ml. of the culture may be calculated by multiplying the number of micrograms read from the calibration curve by the proper factor. This factor will depend upon the dilution of the alcoholic extract. Table I gives the dilutions and the corresponding factor to use. The formula is as follows: micrograms read × factor = tyrothricin per ml. of culture.

In order to test the accuracy of the method under conditions used in the assay procedure, varying amounts of tyrothricin were added to a fully

TABLE II

Accuracy of Hemolytic Assay As Determined by Recovery Tests from Culture Media

No. of trials	Tyrothricin				Recovery	Deviation
	Original culture	Amount added	Total calculated	Found		
	γ per ml.	*γ per ml.*	*γ per ml.*	*γ per ml.*	*per cent*	*per cent*
3		000		342		4
2	342	250	592	627	105	3
2	342	500	842	832	99	1
2	342	750	1092	1136	104	1

TABLE III

Recovery of Tyrothricin from Culture Media by Isolation Procedures

Hemolytic assay (1)	Dry weight (2)	Activity of dry material (3)	Activity recovered (4)
gm. per l.	*gm per l*	*per cent*	*per cent*
1.10	0.90		
2.10	1.76	100	84
0.45	0.37		
0.90	0.73	84	68
0.90	0.80	82	73
0.90	0.83	80	74

grown *Bacillus brevis* culture, and the culture was then tested according to the procedure given. Table II gives a summary of the results obtained.

Isolation Studies—Several *Bacillus brevis* cultures were assayed by the hemolytic method as described. The cultures were then extracted with alcohol and the tyrothricin was isolated and weighed. The dry isolated material, when assayed, appeared to have varying degrees of activity per unit of weight (80 to 100 per cent) as compared to the activity of the pure tyrothricin standard (Column 3, Table III). The per cent of the total activity recovered from the culture media is given in Column 4, Table III.

Additional unpublished data indicate that more activity is recovered when the sample of isolated tyrothricin approaches 100 per cent in activity.

Hemolytic Action of Components—Mixtures of gramicidin and tyrocidine[2] of varying proportions have been prepared, and the hemolytic actions of these mixtures as well as the actions of the two pure components have been determined by the colorimetric procedure as described above. The degree of hemolysis reached at the end of 2 minutes is very nearly constant irrespective of the mixture, within the range of 5 to 35 per cent gramicidin (Table IV). This variation in composition is greater than that thus far found for

TABLE IV

Hemolytic Activity of Various Mixtures of Gramicidin and Tyrocidine Compared with Activity of Tyrothricin

Gramicidin in mixture	Recovery, calculated as tyrothricin, with following amounts of mixture		
	6 γ	8 γ	10 γ
per cent	*per cent*	*per cent*	*per cent*
0	80	75	76
5	87	81	89
10	100	95	98
15	96	107	99
20	100	106	108
25	104	110	103
30	96	99	105
35	100	99	108

natural tyrothricin but the results offer assurance that variation in composition of natural tyrothricin is not likely to affect the accuracy of the method.

SUMMARY

A quantitative method, based upon the hemolytic action of tyrothricin, has been described for the determination of tyrothricin in culture media.

This method is accurate to within 5 per cent, as determined by recovery experiments, and as little as 100 γ of tyrothricin per ml. of culture can be measured. 1 ml. of culture is sufficient for test purposes.

Isolation studies indicate that about 75 per cent of the activity of a culture is obtained by the ordinary extraction procedures.

BIBLIOGRAPHY

1. Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, **73**, 629 (1941).
2. Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **141**, 155 (1941).

[2] The two crystalline compounds were separated from the crude tyrothricin and were recrystallized several times according to the methods of Hotchkiss and Dubos (2).

3. Heilman, D., and Herrell, W. E., *Proc. Soc. Exp. Biol. and Med.*, **46**, 182 (1941).
4. Herrell, W. E., and Heilman, D., *J. Clin. Inv.*, **20**, 583 (1941).
5. Rammelkamp, C. H., and Weinstein, L., *Proc. Soc. Exp. Biol. and Med.*, **48**, 211 (1941).
6. Ponder, E., *Proc. Roy. Soc. London, Series B*, **101**, 193 (1927); **100**, 199 (1926); **95**, 382 (1923).

CHANGES INDUCED BY ANEMIA IN THE BONE MARROW LIPIDS OF CATS*

By R. F. KRAUSE

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 27, 1943)

The bone marrow throughout extrauterine life is the source of the erythrocytes, the granulocytes, and the blood platelets, cellular constituents of the blood which are vital to mammalian existence. The study of a fatty organ thus actively engaged in the production of important cells should present many fundamental problems in lipid chemistry. A review of the literature yielded little information concerning marrow lipids. A few investigators have determined the amounts of total lipid (1–3), phospholipid (1, 2, 4–6), and cholesterol (1, 2, 6) in the marrow of different animals, but these values differ widely. The character of the fatty acids found in marrow has been described by Cheng (7) and Hilditch and Murti (8).

The loss of fat from marrow which has become hematopoietically active, as in anemia, is well known. However, there have been no chemical investigations of how much fat is lost and what changes occur. The objects of this work were (*a*) to determine the qualitative and quantitative composition of the lipids in normal marrow, (*b*) to increase the activity of the marrow by inducing anemia, and (*c*) to determine the resultant changes in the marrow lipids. Marked quantitative changes have been observed; the significance of these is discussed.

EXPERIMENTAL

Source of Material and Method of Sampling—Cats were chosen as the experimental animals because their long bones contain an adequate amount of red (active) marrow. Adult male cats, weighing between 3 and 5 kilos, were anesthetized with ether, the jugular veins were severed, and the animals allowed to bleed to death. The marrow was removed from all the long bones,[1] weighed to the nearest mg., and placed in 95 per cent alcohol.

Analytical Procedures—The marrow samples were extracted with alcohol-

* These studies are taken from a thesis submitted to The University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1942.

[1] The technique used was to remove the epiphyses from both ends of the bony shaft, to slip a piece of rubber tubing connected to a pressure line over one end of the shaft, and to blow the marrow out of the other end of the bone.

ether (3:1) followed by chloroform-methanol (1:1) and the total extracts evaporated to semidryness under reduced pressure (9). The residual paste was extracted repeatedly with petroleum ether and then with chloroform. Small amounts of insoluble material which appeared at this point were combined with the residue from the first extraction and weighed as dry, lipid-free marrow. The petroleum ether-chloroform extract was washed with a saturated sodium chloride solution, and made up to a known volume. Separate aliquots of this extract were used for the determination of free fatty acids (10), free cholesterol (11),[2] and total lipid (gravimetrically). The total lipid sample was saponified in alkaline 95 per cent alcohol. The non-saponifiable material was extracted with petroleum ether, the extract evaporated to dryness, and the residue weighed. Total cholesterol was determined on this fraction. After petroleum ether extraction the solution was acidified, diluted with water to make a 50 per cent alcoholic solution, and the total fatty acids extracted with petroleum ether. Separate aliquots of this petroleum ether extract were used to determine the amount (gravimetrically), the iodine number (14), and the mean molecular weight (10) of the total fatty acids. The alcoholic solution remaining after the final petroleum ether extraction was used for the determination of lipid phosphorus (15) and of choline (16–18). The phospholipid was calculated by multiplying the phosphorus value by 25 (19).

The total weight of dry tissue was calculated by adding the weight of the dry, lipid-free marrow to the amount of total lipid extracted. The water content was calculated by subtracting the total dry weight of marrow from the total wet weight.

Methods for Producing Anemia—Six animals were made anemic by the removal of 25 ml. of blood every other day over a period of 12 days. The cats were anesthetized with ether, and the blood removed by direct cardiac puncture under sterile conditions.

Twelve additional cats were made anemic by the injection daily for 5 days of 14 mg. per kilo of body weight of acetylphenylhydrazine. The drug was dissolved in warm isotonic saline a few minutes before the sterile subcutaneous injection was to be made. During the treatment, the cats became jaundiced, nauseated, and exhibited a slight anorexia. If these symptoms were exaggerated, the injection was omitted for a day.

[2] Instead of precipitating the phospholipid from the total lipid fraction, as suggested by Kelsey (11), the phospholipid and cholesterol were removed from the lipid fraction by digitonin precipitation (12). The precipitate was then boiled with benzene and extracted with petroleum ether. Phospholipid was removed from this extract by the Bloor procedure (13), and the supernatant fluid was used for the determination of free cholesterol.

DISCUSSION

Lipid Changes in Marrow during Anemia—The total lipid of normal marrow averaged 5.2 gm. in 10 gm. of wet marrow.[3] The concentration of lipid in marrow exceeds that normally found in any other organ of the body; the next highest is in brain, which has about 13 per cent, wet weight (16). However, some pathological tissues have a higher fat content than marrow; for example, fatty livers have been reported to contain as high as 65 per cent total lipid, wet weight (20).

From the data in Table I it can be seen that during anemia the total lipid concentration decreased by about 50 per cent. The major portion of the loss was in the neutral fat fraction; *e.g.*, in cats with hematocrit values of about 9 per cent, 10 gm. of wet marrow lost 3.5 gm. of neutral fat. However, all other fractions of the total lipid increased. The neutral fat constitutes so large a part of the total lipid of the marrow that the 55 per cent decrease in this fraction could account for the decrease in the total lipid despite the increased percentages of the other constituents, if the volume remained constant.

The loss of neutral fat from marrow may proceed by either selective or non-selective removal of the available fat. If there were a selective removal of fat during anemia, this loss should cause changes in the character of the residual total fatty acids, since the neutral fat fraction makes up 90 to 97 per cent of the total lipid. No such changes were observed; the iodine number remained approximately 60 and the mean molecular weight 280 (Table II). Therefore, it seems reasonable to suppose that the loss of neutral fat from marrow is non-selective. This finding offers additional evidence for the view held by Longenecker (21) and recently restated by MacLachlan *et al.* (22) that "The mobilization of the body lipids probably proceeds by a non-selective utilization of the available fat."

The rôle played by neutral fat in the hematopoietic functions of marrow is not clear. Perhaps, as Sabin (23) has suggested, the removal of neutral fat makes room for the deposition of material necessary for cell construction. It is equally difficult to assign a function to the unmobilized portion; it may also have some use *per se* in the construction of red blood cells, since they contain approximately 0.04 per cent, wet weight, of neutral fat (24).

Although the total lipid of marrow decreases during anemia, phospholipid, free fatty acids, cholesterol, and cholesterol-free non-saponifiable material (Table I) all increase by approximately 50 per cent. The relation between these various fatty materials remains remarkably constant during

[3] This is approximately the amount of marrow in a cat.

TABLE I

Balance Sheet of Chemical Components of Red Marrow from Normal and Anemic Cats (Expressed in Per Cent of Wet Weight)

Animal No	Total lipid	Water	Residue*	Free fatty acids	Phospho-lipid	Non-saponifiable material	Total cholesterol	Free cholesterol	Hematocrit value
				Normal					
1	57 5	22.4	19.9	0.22	0.54	0.26	0.07	0.06	
3	66 5	25.8	7.6	0.33	0.53	0.48	0.07	0.06	
4	59.3	27.9	11.9	0.32	0.48	0.22	0.06	0.04	
5	45.7	39.8	14.7	0.55	0.73	0.42	0.12	0.08	
6	53 8			0.30	0.53	0.59	0 06	0.05	
7	60 8	32.6	6 6	0.30	0.61	0.33	0.08	0.05	
8	46.1	35 6	18.3	0.17	0.34	0.22	0.05	0.04	
9	51.6	38.2	10.0	0 45	0.72	0.25	0.12	0.08	
11	39 8	45.0	19.9	0.56	0.97	0.57	0.12	0.08	
12	57 0	32.8	7 6	0.27	0.55	0.40	0.07	0.03	
14	44.2	55 0	11.6	0.22	0.70	0.23	0.12	0 08	
15	29 0	51.0	20.0	0.39	0.73	0 24	0.09		
16	55.0	27 6	16 5	0.53	1.19	0.36	0.15		
18	45 5	38 0	16.7	0.27	0 64	0.22	0.11	0.08	
19	54.1	36.4	9.8	0.32	0.74	0.18	0.11	0.07	
20	60 6	33.3	5 8	0.22	0.43	0.07	0.06	0.05	
21	64.0	29.3	7.0	0.19	0.53	0.10	0.05	0.04	
22	65.5	23.0	11 3	0.19	0.46	0.12	0.05	0.04	
23	27 8	54 0	18.3	0 33	0 63	0 28	0 17	0.07	
Average deviation	9.0	8 1	4 3	0.09	0.14	0.11	0.03	0.02	
Standard deviation	10.9	9.7	4.8	0.10	0.19	0.14	0.03	0.02	
Average	51 6	35.9	13 0	0 31	0.63	0.28	0.09	0.06	
				Anemic					
H-1	18.90	65.0	16.10	0.36	0.93	0.62	0.15	0.15	25
H-2	59.00	32.8	8.20	0.26	0.49	0.28	0.09	0.07	44
H-3	32.00	56.2	11.8	0.43	0.97	0.25	0.12	0.12	26
A-4	17 5	66.5	16.0	1.42	1.12	0.60	0.17	0.16	28
A-5	24 6	59.5	15 9	0 18	0.88	0.74	0.14	0.11	22
A-6†	12.3	70.3	17 4	0.57	1.03	0.48	0.14	0.10	12
A-7†	29.6	44.0	26.4	0.35	1.05	0.35	0.12	0.06	19
A-9†	15.6	58.0	26.4	0.27	0.79	0.19	0.07	0.04	9
A-10	26.4	58.0	15.6	0.34	1.00	0.30	0.12	0.09	32
A-11	16.8	65.5	17.7	0.33	1.09	0.45	0.16	0.09	15
A-12	12.0	70.0	18.0	0.41	1.00	0.37	0.14	0.08	15
A-13	26.0	60.0	14.0	0.43	1.08	0.31	0.15	0.06	18
A-14†	16.4	67.5	16.1	0.28	1.03	0.33	0.14	0.06	13

TABLE I—*Concluded*

Animal No.	Total lipid	Water	Residue*	Free fatty acids	Phospho-lipid	Non-saponifiable material	Total cholesterol	Free cholesterol	Hematocrit value
				Anemic—*Concluded*					
A-15	34.0	53.6	12.4	0.54	0.99	0.45	0.14	0.08	31
H-16†	10.3	72.7	17.0	0.39	1.40	0.24	0.16	0.14	14
H-17	34.6	53.5	11.9	0.31	0.84	0.37	0.10	0.09	21
H-18	32.6	55.7	11.7	0.33	1.12	0.36	0.16	0.10	21
Average deviation.	9.1	6 8	3.2	0.15	0.14	0.12	0.02	0.02	
Standard deviation	11.1	4.6	8.4	0.16	0.19	0.15	0 03	0.03	
Average	24.5	59.5	16.0	0.43	0.99	0.40	0.14	0.09	

A = anemia produced by acetylphenylhydrazine. H = anemia produced by bleeding.

* Dry, lipid-free marrow.

† Choline-treated.

anemia. Further evidence (Table II) of this constancy is seen in the average phospholipid to cholesterol ratios (normal 7.3; anemic 7.4) and in the average ratios of the molar concentrations of choline to phosphorus (normal 0.87; anemic 0.89). This constancy indicates that each of the lipid fractions is equally important in the building of blood cells. The probable reason for their increase in marrow during anemia is that more material is needed for the all-out production of blood cells.

Phospholipid and cholesterol may be incorporated directly into the blood cells, since these cells have been shown to contain 500 mg. per cent of phospholipid (19) and about 100 mg. per cent of cholesterol (24). Sinclair (25), using elaidic acid, and Hahn and Hevesy (26) using radioactive phosphorus have shown that phospholipid is not formed in the red blood cell after it has entered the blood stream. These findings seem to afford evidence that the phospholipid of marrow is used to build blood cells. The similarity of the ratios of phospholipid to cholesterol in normal and anemic marrow (average 7) and in red blood cells (average 5) calculated from data of Hunter (19) and Erickson *et al.* (24) suggests that the cholesterol in marrow is also used to build cells. The increase of these compounds in marrow during anemia may be attributed to the need for the increased production of cells.

It is interesting to note that bone marrow like such other tissues as muscle (27), corpus luteum (28), mammary gland (29), and tumor (30) shows an increase in its phospholipid content as the tissue increases in

TABLE II

Physical and Chemical Constants of Lipids of Red Marrow from Normal and Anemic Cats

	Animal No.	Iodine No.	Mean mol. wt. of fatty acids	Phospholipid / Cholesterol ratio	Choline / Phosphorus ratio
Normal	1	65	292	7.9	0.77
	3	70	298	7.8	0.79
	4	57	294	6.4	0.92
	5	70	260	7.8	0.84
	6	61	284	8.3	1.00
	7	76	298	8.0	0.72
	8	59	274	6.9	0.75
	9	59	293	6.0	0.85
	11	61	296	6.3	0.75
	12	64	272	8.2	0.74
	14	73	280	5.8	0.90
	15	58	316	8.2	0.82
	16	62	299	9.1	1.00
	18	52	286	5.8	1.00
	19	53	294	6.6	0.94
	20	53	286	6.8	0.94
	21	59	290	10.0	0.87
	22	61	293	9.1	0.92
	23	59	284	3.5	0.99
Average		62	289	7.3	0.87
Anemic	H-1	61	270	6.13	1.10
	H-2	62	280	5.20	0.79
	H-3	59	290	8.00	1.06
	A-4	64	270	6.65	0.94
	A-5	62	260	6.32	1.10
	A-6*	64	280	7.40	0.85
	A-7*	62	286	8.20	0.84
	A-9*	58	272	12.0	0.50
	A-10	55	280	7.75	0.80
	A-11	57	270	7.00	0.50
	A-12	55	268	6.60	0.80
	A-13	57	310	7.40	1.03
	A-14*	59	250	7.23	1.20
	A-15	57	270	6.93	0.90
	H-16*	58	270	8.40	0.80
	H-17	70	268	8.05	0.92
	H-18	58	280	7.10	0.94
Average		60	275	7.43	0.89

A = anemia produced by acetylphenylhydrazine. H = anemia produced by bleeding.

* Choline-treated.

activity. This observation falls in line with the view held by Bloor that there is a relationship between the physiological activity of tissues and their phospholipid content.

The ratio of free to bound cholesterol in the marrow of normal and anemic cats is about 2.4. This ratio is like that found in other tissues, and indicates the presence of little cholesterol ester (31). Bloor, Okey, and Corner (28) and Haven (30) have shown that the cholesterol ester content varies inversely with the physiological activity of the organ. In this experiment, increased activity of the bone marrow has produced little change in the cholesterol ester content.

An examination of Table II shows that the average molar ratios of choline to phosphorus in both normal and anemic marrow are about 0.9. In other words, the various fractions of phospholipid have increased in the same proportion. This ratio (0.9) is of interest because it indicates the presence of large amounts of choline-containing phospholipids and correspondingly small amounts of cephalin. The high value of this ratio is unusual and presents an interesting anomaly. The assumption has previously been made that many of the variations discovered in the amounts of neutral fat, cholesterol, etc., are due to the demands of hematopoiesis, and that certain substances, *e.g.* phospholipids, are brought to the marrow from other locations in the body. The phospholipids of the red cells (32) and platelets (33) are approximately 60 per cent cephalins. Consequently, according to the hypothesis, it would be expected that cephalin would be mobilized and furnished in larger amounts than lecithin to the marrow. Such is evidently not the case. However, it is possible that in active hematopoiesis the demands for cephalin are sufficiently acute to reduce the amount present in the marrow to the low value observed.

The small increase of the non-saponifiable fraction during anemia parallels that of the other lipid fractions. This increase is not due to cholesterol alone, for other components make up about 50 per cent of this fraction or about 20 mg. per 10 gm. of wet marrow. Little is known about the composition of this material, other than that it contains batyl alcohol (34). According to the yield obtained by Holmes *et al.* (34) this alcohol would account for one-half of the cholesterol-free, non-saponifiable fraction. Small amounts of vitamin A may also be present in this fraction.[4] The possible rôle of some substance or substances in the non-saponifiable fraction of marrow in stimulating this tissue has been shown by Watkins and Giffin (35) and later by Marberg and Wiles (36).

Possible Explanation for Lipemia after Hemorrhage—The lipemia after acute hemorrhage in man and in animals has been explained in various ways (37). Bloor (38) postulated that there is a displacement of fat

[4] McCoord, A. B., unpublished data.

from the blood-forming tissues into the blood in a greater quantity than the normal mechanism can dispose of at once. Our results support this explanation in part, since neutral fat is lost from marrow during anemia. However, the increase of the other lipid fractions in marrow may be accounted for by a mobilization of these materials from other tissues to marrow via the plasma. Therefore, the lipemia may result from the transport of lipids both to and from the marrow during anemia.

Relationship between Water and Fat—Red marrow from normal cats was found to contain 35.9 per cent water and 51.6 per cent total lipid. In anemia the lipid content decreased to 24.5 per cent and the water content increased to 59.5 per cent (Table I). It is evident that an inverse relationship exists between the water and fat content of marrow (Fig. 1). In

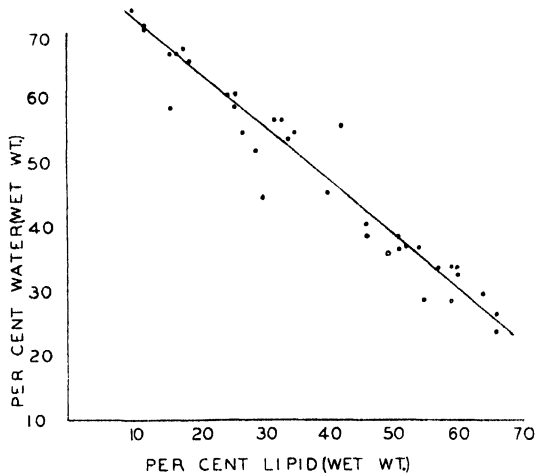


FIG. 1. The relationship between water and lipid content of marrow

view of the large amount of fat in marrow and the changes undergone during anemia, it seemed worth while to estimate whether fat is deposited in the marrow with an associated quantity of water. If water and lipid are calculated as per cent of lipid-free marrow, any water associated with the lipid would serve to increase the calculated water percentage as the lipid increased. Since there was little change in the water percentages (dry, lipid-free basis) with various amounts of lipid, it seems logical to conclude that water does not accompany the deposition of the lipid in marrow.

SUMMARY

1. In anemia, bone marrow loses total lipid, owing to a reduction in the neutral fat fraction. The phospholipid, free fatty acid, cholesterol, and cholesterol-free non-saponifiable fractions increase in anemia.

2. The ratios of free to bound cholesterol, of phospholipid to cholesterol, and of the molar concentration of choline to that of phosphorus show no significant change during anemia.

3. There is no significant change in the iodine number or mean molecular weight of the fatty acids of marrow during anemia.

4. The lipemia after acute hemorrhage may be due to the transport of lipids to and from the marrow.

5. An inverse relationship exists between the water and lipid content of marrow.

BIBLIOGRAPHY

1. Nerking, J., *Biochem. Z.*, **10**, 167 (1909).
2. Jastrowitz, H., *Z. ges. exp. Med.*, **55**, 523 (1927).
3. Huggins, C., McFadyen, J., and Wiege, E., *Anat. Rec.*, **76**, 309 (1940).
4. Glikin, W., *Biochem. Z.*, **4**, 235 (1907).
5. Otolski, S. W., *Biochem. Z.*, **4**, 124 (1907).
6. Beumer, H., and Burger, M., *Z. exp. Path. u. Therap.*, **13**, 367 (1914).
7. Cheng, L. T., *Z. physiol. Chem.*, **201**, 209 (1931).
8. Hilditch, T. P., and Murti, K. S., *Biochem. J.*, **34**, 1299 (1940).
9. Haven, F. L., and Levy, S. R., *J. Biol. Chem.*, **141**, 417 (1941).
10. Gortner, W. A., Thesis, The University of Rochester (1940).
11. Kelsey, F. E., *J. Biol. Chem.*, **127**, 15 (1939).
12. McEwen, H. D., and MacLachlan, P. L., *Proc. Soc. Exp. Biol and Med.*, **48**, 195 (1941).
13. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
14. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
15. Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, **86**, 671 (1930).
16. Taylor, J. D., Thesis, The University of Rochester (1940)
17. Beattie, F. J. R., *Biochem. J.*, **30**, 1554 (1936).
18. Kirk, E., *J. Biol. Chem.*, **123**, 623 (1938).
19. Hunter, F. E., *J. Biol. Chem.*, **144**, 439 (1942).
20. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **116**, 663 (1936).
21. Longenecker, H. E., *J. Biol. Chem.*, **129**, 13 (1939).
22. MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D., *J. Biol. Chem.*, **143**, 473 (1942).
23. Sabin, F. R., *Physiol. Rev.*, **8**, 191 (1928).
24. Erickson, B. N., Williams, H. H., Bernstein, S. S., Avrin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, **122**, 515 (1937–38).
25. Sinclair, R. G., *J. Biol. Chem.*, **115**, 211 (1936).
26. Hahn, L., and Hevesy, G., *Nature*, **144**, 72 (1939).
27. Bloor, W. R., *J. Biol. Chem.*, **119**, 451 (1937).
28. Bloor, W. R., Okey, R., and Corner, G. W., *J. Biol. Chem.*, **86**, 291 (1930).
29. Bloor, W. R., and Snider, R. H., *J. Biol. Chem.*, **107**, 459 (1934).
30. Haven, F. L., *Am. J. Cancer*, **29**, 57 (1937)
31. Bloor, W. R., *Physiol. Rev.*, **19**, 557 (1939).
32. Kirk, E., Annual review of biochemistry, Stanford University, **9**, 115 (1940).
33. Erickson, B. N., Lee, P., and Williams, H. H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **123**, p. xxxiv (1938).

34. Holmes, H. N., Corbet, R. E., Geiger, W. B., Kornblum, N., and Alexander, W., *J. Am. Chem. Soc.*, **63**, 2607 (1941).
35. Watkins, C. H., and Giffin, H. Z., *J. Am. Med. Assn.*, **95**, 587 (1930).
36. Marberg, C. M., and Wiles, H. O., *J. Am. Med. Assn.*, **109**, 1965 (1937).
37. Muller, G. L., *Medicine*, **9**, 119 (1930).
38. Bloor, W. R., *J. Biol. Chem.*, **49**, 201 (1921).

THE INACTIVATION OF TRYPSIN BY ULTRAVIOLET RADIATION*

By FRANK VERBRUGGE†

(From the Biophysical Laboratory of the Department of Physics, University of Missouri, Columbia)

(Received for publication, May 12, 1943)

The efficiency of ultraviolet light in the inactivation of enzymes has been determined for pepsin (1), for urease (2, 3), and at two wave-lengths for trypsin (4). The quantum yields obtained with pepsin (*cf.* (3)) vary from 0.0003 to 0.0014 molecule per quantum and, in general, increase with decreasing wave-length. A similar wave-length response occurs with urease (3); the quantum yields vary from 0.0007 at 2800 Å. to 0.009 at 1860 Å. Over the same wave-length range, Kubowitz and Haas (2) report an almost constant quantum yield. For trypsin (4) yields of 0.017 and 0.016 are reported at 2537 and 2804 Å., respectively.

Different methods have been used to determine the extent of the inactivation in all these cases. For urease, the production of ammonia (3) and the rate of production of carbon dioxide (2) were used; for pepsin, the colorimetric method of Folin and Ciocalteu (5) as applied to protein hydrolysis (6). The method of Grassmann and Heyde (7) with benzoyl-argininamide as substrate was used for trypsin. It is of interest, therefore, to determine whether the nature of the enzyme substrate and the methods of determining the extent of inactivation affect the values of the quantum yields obtained. Inactivation of an enzyme molecule by ultraviolet light results from the absorption of quanta by active centers; if these centers are independent of each other in their enzyme action, all of them must be inactivated in order to produce complete inactivation of each molecule. That is, the loss of activity as measured experimentally will be directly proportional to the true extent of the inactivation only if the loss of activity at one locus does not affect the activity of the remaining active centers. The use of different methods of determining the extent of inactivation with several substrates differing in complexity is essential to answering this question of independence of action. Furthermore, a similarity in wave-length dependence in quantum yields as measured by the various methods would tend to support the hypothesis that the active

* This manuscript is part of a dissertation presented to the Faculty of the Graduate School of the University of Missouri in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Now at Carleton College, Northfield, Minnesota.

centers are alike in their action and, therefore, probably alike in their chemical constitution.

EXPERIMENTAL

Preparation of Trypsin—The crystalline trypsin was obtained by the method of Kunitz and Northrop (8). Its purity was determined by its rate of hydrolysis of benzoylargininamide. The activity coefficient was 0.038, in close agreement with results previously reported (9).

Absorption Spectrum—The absorption spectrum of the trypsin was determined in M/15 phosphate buffer solution, pH 4.5. The trypsin concentration was 2.5×10^{-5} M, based on a molecular weight of 36,500. The spectrum is shown in Fig. 1. This agrees with that previously reported

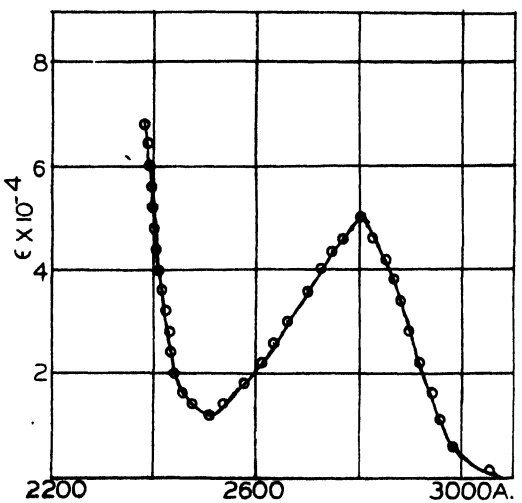


FIG. 1. Absorption spectrum of trypsin

(4) in the wave-length positions of the maximum and minimum absorption and also in the value of the molecular extinction coefficient (50,000) at the position of maximum absorption at 2800 Å. However, $\epsilon_{min.}$ at 2506 Å. has a value of only 12,000, in contrast with the reported value of 29,000. The molecular extinction coefficient is here defined in the usual manner,

$$\epsilon = \frac{1}{Cl} \log_{10} \frac{I_0}{I_t}$$

where C is the concentration in moles per liter, l is the length of path in cm. through the solution, and I_0 and I_t are the incident and transmitted light intensities, respectively. The absorption measurements were made by Dr. V. R. Ells in the University of Missouri Spectrographic Laboratory. A Hilger medium quartz spectrograph and a Spekker photometer were used.

Irradiation Technique—A water-cooled capillary mercury vapor lamp operating at atmospheric pressure served as the source of the ultraviolet radiation. The crystal quartz monochromator had a Young type of mounting, as previously described (3); its rectangular exit slit had an area of 10.08 sq. mm. The intensity of the radiation was measured by a surface type of vacuum thermopile with Ag-Bi elements, calibrated against a United States Bureau of Standards Lamp C-241. A galvanometer scale deflection of 1 cm. represented an energy flow of 25.2 ergs per second. A rectangular, fused quartz irradiation cell, 4 × 10 × 40 mm., was used for all irradiation trials. It was placed directly in front of the thermopile face. The trypsin solution, at a concentration of 2.5×10^{-5} M and pH 4.5, was stirred continuously by a motor-driven quartz rod during irradiation.

The energy absorbed by the active trypsin in the solution was calculated from the incident energy by taking into account a variety of factors. These included reflection and absorption losses by the front face of the irradiation cell, absorption by the buffer medium, and absorption by the inactivated trypsin. Since the concentration of the inactive trypsin is continually increasing during irradiation, an average value for its absorption was obtained by graphical integration.

Activity Determinations—Three substances were used as substrates for the native and irradiated trypsin solutions. These were hemoglobin, casein, and benzoylargininamide; the last was prepared by Mr. A. D. McLaren in the form of its hydrochloride (*cf.* (10)). The hemoglobin solution was prepared from beef blood by the method of Anson (6). The casein solution was prepared from casein (Merck, according to Hammarsten), kindly furnished by Dr. D. Mazia of the Department of Zoology. Curves representing the relationship between the extent of hydrolysis and enzyme concentration were determined for each of the substrates for each experimental method employed; these curves were used as reference curves for determining the extent of the inactivation of the irradiated trypsin.

Benzoylargininamide—The benzoylargininamide solution was prepared at a concentration of 0.04 M in M/15 phosphate-NaOH buffer at pH 11.5; 0.5 cc. of the trypsin solution was added to 0.5 cc. of the substrate solution and the mixture was incubated for 1 hour at 35° and pH 7.5. The extent of hydrolysis was determined by the method of Grassmann and Heyde ((7), *cf.* (4)), which consists of a titration of the COOH groups liberated during hydrolysis, the titration being carried out in a 90 per cent alcoholic solution. The per cent inactivation was determined by comparison with the corresponding reference curve.

Hemoglobin—Inactivation measurements with hemoglobin as substrate were made by the colorimetric method (6) and the formol titration method. For the former method, the 2.5×10^{-5} M enzyme solution was diluted to

one-fiftieth after irradiation. 1 cc. of this solution was added to 5 cc. of the hemoglobin solution and digested at pH 7.5 for 15 minutes. Its color value was compared with a control solution and the per cent inactivation determined.

In order to be able to compare the results obtained with hemoglobin more directly with those of benzoylargininamide, inactivation measurements were made by titration also. The color of the hemoglobin makes a visual end-point titration impossible. Electrometric titration in 90 per cent alcohol is also unreliable. The titrations were, therefore, carried out electrometrically in the presence of approximately 6 per cent formaldehyde. For this method, the hemoglobin solution was prepared in the same manner as for the colorimetric method, except that the alkali concentration was decreased to about 0.05 M. To 5 cc. of this solution at pH 7.9 was added 0.5 cc. of the 2.5×10^{-5} M enzyme solution. The mixture was incubated for 20 minutes at pH 7.7. Enough 0.2 N NaOH was added to a blank to bring it to pH 8.5. 1 cc. of 38 per cent formaldehyde was added and the solution was titrated with 0.02 N NaOH to pH 9.0. The titration was made electrometrically with a Beckman pH meter, with shielded electrodes so that the titrations could be made externally. As much 0.2 N NaOH was added to each of the control and irradiated solutions as was added to the blank and the formol titrations were then completed in the same manner. The relative digestion values were obtained by correcting for the blank, and the trypsin concentration of the irradiated sample determined by comparison with the reference curve.

Casein—The extent of trypsin inactivation as measured by the digestion of casein was determined by two methods, the colorimetric method and the Grassmann-Heyde method. The colorimetric method was similar to that used for hemoglobin. The trypsin solution was diluted to one-fortieth and 1 cc. of this solution added to 5 cc. of 5 per cent casein solution in 0.1 M phosphate buffer at pH 7.8. The mixture was incubated for 15 minutes at pH 7.6 and the concentration of the active trypsin remaining after irradiation determined colorimetrically.

The titration method with casein was similar to that used for benzoylargininamide. To 3 cc. of the 5 per cent casein solution was added 0.5 cc. of enzyme solution, and, after incubation for 90 minutes, at pH 7.5, the extent of hydrolysis was determined by titration with alcoholic KOH.

Results

The efficiency of the ultraviolet radiation in destroying trypsin activity as measured by the various methods is summarized in Table I. In general, the quantum yield (molecules inactivated per quantum absorbed) in the region of minimum absorption (2537 Å.) and at shorter wave-lengths

is twice that obtained at longer wave-lengths. This ratio is independent of the type of substrate and the experimental method. The absolute values of the quantum yields obtained with casein agree with those obtained with benzoylargininamide and (except at 2537 Å.) with hemoglobin when the formol titration method is used. The colorimetric method with hemoglobin gives a value which is from 3 to 4 times as large as that obtained by the other methods. It appears, therefore, that the Anson colorimetric method, as applied to hemoglobin, is not a true measure of tryptic activity when inactivation has been produced by radiation.

TABLE I

Quantum Yields for Trypsin Inactivation (Molecules Inactivated per Quantum Absorbed)

Substrate	Method	Wave-length	No. of determinations	Average quantum yield
		Å.		
Benzoylarginin-amide	Grassmann-Heyde	2399	11	0.025 ± 0.001
		2537	13	0.024 ± 0.002
		2650	8	0.012 ± 0.001
		2804	11	0.013 ± 0.001
Hemoglobin	Colorimetric	2399	10	0.087 ± 0.002
		2537	9	0.103 ± 0.003
		2537*	9	0.109 ± 0.006
		2650	12	0.053 ± 0.002
		2804	10	0.048 ± 0.001
"	Formol	2399	5	0.027 ± 0.001
		2483	7	0.027 ± 0.001
		2537	8	0.040 ± 0.002
		2804	6	0.015 ± 0.001
Casein	Colorimetric	2537	5	0.019 ± 0.001
		2804	8	0.011 ± 0.001
"	Grassmann-Heyde	2537	8	0.026 ± 0.002
		2804	8	0.012 ± 0.001

* Concentration = 10^{-4} M.

The quantum yield at 2537 Å. with hemoglobin as substrate is somewhat higher than that obtained at shorter wave-lengths. This increased yield is obtained with both the colorimetric and the formol methods. That this is not due to impurities in the radiation is shown by the fact that the same quantum yield was obtained when the trypsin was irradiated at a higher concentration, so that the absorption at 2537 Å. was comparable to that at neighboring wave-lengths. The range over which this higher quantum yield is obtained is not large; the yield at 2483 Å. agrees with that at 2399 Å.

DISCUSSION

The quantum yields for trypsin are higher than those reported for urease and pepsin. There appears to be no direct correlation between quantum yield and molecular weight, since pepsin and trypsin have comparable molecular weights. The yields increase with shorter wave-lengths; the increase for trypsin appears quite suddenly in the region of minimum absorption; for pepsin and urease the increase is gradual. This difference is probably due to the fact that the absorption minimum is much more marked in the case of trypsin; this means that the absorbing regions become more nearly distinguishable and differences in quantum efficiency will appear rather sharply.

The quantum efficiency at 2537 Å. is twice that at 2804 Å. Uber and McLaren (4) obtained about the same yield at these two wave-lengths. The difference again appears to be due to the difference in the absorption coefficient at 2500 Å. for the trypsin samples used. The value of $\epsilon_{min.}$ for their trypsin sample was 29,000, compared with 12,000 for the trypsin used in the present investigation.

The activity of the irradiated trypsin decreases exponentially as a result of irradiation. Such a relation indicates that a "one hit" reaction is involved. It does not mean, however, that a single quantum hit inactivates the entire enzyme molecule. This would imply the existence of only one active center in the molecule, which is almost certainly not true. The exponential inactivation curve shows rather that the active groups are independent of each other in their enzyme action and that a single quantum is sufficient to inactivate any one of these groups. This independence of action does not depend upon the size of the substrate molecule, since the exponential inactivation was obtained both with the benzoylargininamide and with the protein substrates. A quantum yield of 0.012 or 0.024 does not mean, therefore, that only 1 out of 80 or 40 quanta, respectively, is effective in inactivating the trypsin molecule. The pepsin acetylation experiments of Herriott and Northrop (11, 12) show that the number of active centers may be twenty-five or more. If a comparable number occurs in trypsin, the quantum efficiency based on the number of active centers is actually much higher than is indicated by the "molecular" quantum yield, and approaches unity for the shorter wave-lengths.

When the trypsin inactivation is measured colorimetrically with hemoglobin, a higher quantum yield is obtained than with the other methods. Since this higher yield is not obtained with the formol titration method, it is a characteristic of the experimental method, not of the hemoglobin substrate. By means of the Anson colorimetric method the number of tyrosine residues present in a 0.2 N trichloroacetic acid filtrate is determined. This concentration of acid will precipitate both the denatured

hemoglobin and the larger split-products of hydrolysis. If one assumes that the several active groups in the trypsin molecule simultaneously attack the many susceptible bonds in a hemoglobin molecule, a fairly large number of smaller split-products will result. In the irradiated trypsin, however, many of the enzyme molecules have a large percentage of their active centers inactivated. Hydrolysis of hemoglobin by such a molecule will result in larger split fragments, fewer of which will appear in the filtrate. An apparently higher quantum yield will result.

On the other hand, the degree of trypsin inactivation as measured by casein hydrolysis is the same with the colorimetric method as with the titration method. This means that very nearly the same fraction of hydrolysis fragments appears in the trichloroacetic acid filtrate with irradiated trypsin as with unirradiated.

A second characteristic of the measurements with hemoglobin is the higher yield obtained at 2537 Å. by both the colorimetric and the formol titration methods. The significance of this result is uncertain. Of the amino acids present in the trypsin molecule, only phenylalanine and cystine have selective absorption in this region; that of cystine is quite weak. It is possible that the presence of either of these residues in active form is particularly important in the digestion of hemoglobin.

The writer is indebted to Dr. Fred M. Uber for his advice and direction throughout this investigation.

SUMMARY

1. An absorption spectrum for crystalline trypsin has been obtained and has been compared with the spectrum previously reported.

2. Quantum yields for the inactivation of trypsin have been measured with three substrates, benzoylargininamide, hemoglobin, and casein. Measurements were made colorimetrically and by titration for liberated COOH groups.

3. Quantum yields have been obtained at 2399, 2483, 2537, 2650, and 2804 Å. The yields at the three shorter wave-lengths are approximately twice as great as at 2650 and 2804 Å.

4. The quantum yields as measured by benzoylargininamide and casein agree and are independent of the method of determination. For hemoglobin, the titration method gives results which agree with those with the other substrates; the colorimetric method gives values which are considerably higher. These results indicate that the Anson colorimetric technique is not generally applicable to irradiated enzymes.

5. The inactivation of trypsin follows a simple exponential curve. From this it is concluded that the active centers in the trypsin molecule are independent in their action.

BIBLIOGRAPHY

1. Gates, F. L., *J. Gen. Physiol.*, **18**, 265 (1934).
2. Kubowitz, F., and Haas, E., *Biochem. Z.*, **257**, 337 (1933).
3. Landen, E. W., *J. Am. Chem. Soc.*, **62**, 2465 (1940).
4. Uber, F. M., and McLaren, A. D., *J. Biol. Chem.*, **141**, 231 (1941).
5. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
6. Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
7. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1923).
8. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936).
9. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **138**, 243 (1941).
10. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939).
11. Herriott, R. M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 35 (1934).
12. Herriott, R. M., *J. Gen. Physiol.*, **19**, 283 (1935).

ADRENOCORTICOTROPIC HORMONE

By CHOH HAO LI, HERBERT M. EVANS, AND MIRIAM E. SIMPSON

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, May 25, 1943)

Of six hormones[1] identified in the anterior hypophysis, the adrenocorticotropic hormone is least known. Since the observation of Smith (6), only five publications have appeared (7–11) which give methods that may be used to secure a solution concentrated in adrenocorticotropic activity. No agreement is found among these workers as to the chemical nature of the hormone. In order to establish the biological characteristic of a hormone from a complex source such as the pituitary, it must first be isolated in pure form judged both by *chemical* and *biological* data. The present paper presents a method[2] for the isolation of the adrenocorticotropic hormone which is freed from other active contaminants, and behaves chemically as a single substance.

Method of Isolation

1. Acid-Acetone Extract of Fresh Gland—2 kilos of fresh sheep glands were ground twice in an electric meat grinder with 1 liter of water. The ground gland was then poured into 8 liters of acetone containing 200 cc. of concentrated HCl (C.P. grade, sp. gr. 1.19). The mixture was stirred for 1 hour and filtered by suction. The residue was washed in the filter with 1 liter of 80 per cent acetone. This procedure was carried out at room temperature; all succeeding steps were conducted in a cold room at 2–3°.

To the clear brown filtrate and washings, 32 liters of chilled acetone (−5°) were added. The mixture was allowed to settle overnight. The supernatant was siphoned off and the precipitate was washed two or three times with cold acetone on a suction filter. The precipitate, designated as *the acid-acetone powder*, served as the starting material for the isolation of the adrenocorticotropic and lactogenic hormones. It may be stored in a desiccator until needed. From 2 kilos of sheep pituitaries, about 35 gm. of acid-acetone powder were obtained.

2. Na_2HPO_4 Extract of Acid-Acetone Powder—The acid-acetone powder (50 gm.) was next extracted with 1 liter of 0.10 M Na_2HPO_4 for 6 hours with constant stirring and then centrifuged; as some floating material remained

[1] Of these hormones, lactogenic, interstitial cell-stimulating, follicle-stimulating, growth, thyrotropic, and adrenocorticotropic hormones, only the first two have been previously isolated in pure form (1–5).

[2] For a brief account of the method see (12).

after prolonged centrifugation of this first extract, it was filtered after centrifugation. The residue was reextracted twice with 600 cc. of the same solvent for 12 hours each time. The residue showed no adrenocorticotropic or lactogenic activities and was therefore discarded.

The combined extracts were then brought to half saturation of $(NH_4)_2SO_4$ by the addition of the solid salt. The mixture was allowed to stand for 4 hours and then centrifuged. The supernatant was devoid of hormonal activities and was discarded.

3. Separation of Lactogenic and Adrenocorticotropic Hormones—The $(NH_4)_2SO_4$ precipitate was then dissolved in about 300 cc. of water and dialyzed until salt-free. The dialyzed, insoluble material was then centrifuged off and discarded. The clear supernatant was made to pH 3.0 with 1 M HCl, and a saturated NaCl solution was added dropwise with mechanical stirring until the concentration of NaCl became 0.06 saturated. The precipitate was then centrifuged and saved for the isolation of the lactogenic hormone (3).

4. Ammonia Treatment—The 0.06 saturated NaCl solution was next brought to 0.50 saturation by the addition of saturated NaCl solution. After the solution had stood for 4 to 5 hours, the precipitate was removed by centrifugation and the supernatant was discarded. The 0.50 saturated NaCl precipitate was dissolved in about 100 cc. of water and 50 cc. of ammonia (sp. gr. 0.90) were added. The solution containing ammonia was allowed to stand at room temperature for 10 to 15 hours; it was then poured into 1500 cc. of chilled acetone (−5°).

The acetone supernatant was decanted and centrifuged off; the precipitate was dissolved in water (about 150 cc. of solution) and dialyzed until free from acetone and electrolytes.

5. $(NH_4)_2SO_4$ Fractionation—If some precipitate appeared during the dialysis, it was removed before the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution to the dialyzed material. The soluble, 0.5 saturated $(NH_4)_2SO_4$ substance had no activity and was removed by centrifugation. The precipitate was dissolved in 120 cc. of phosphate buffer (pH 7.52) with an ionic strength of 0.10 and the solution was brought to 0.2 saturation by the addition of saturated $(NH_4)_2SO_4$. The 0.2 saturated $(NH_4)_2SO_4$ precipitate was centrifuged off and discarded; $(NH_4)_2SO_4$ was added until the supernatant became 0.4 saturated.

The 0.2 to 0.4 saturated $(NH_4)_2SO_4$ precipitate was again dissolved in buffer of pH 7.52 and the $(NH_4)_2SO_4$ precipitation repeated two more times. The insoluble material of the last 0.4 saturated $(NH_4)_2SO_4$ was then dissolved in water and dialyzed first against water and then buffer of pH 7.52.

6. Heat Treatment—The solution after dialysis at pH 7.52 was diluted

with the buffer until it contained 0.5 per cent protein and was put into a water bath at 100° for 2 to 3 hours. There appeared but little coagulum during heating and this was removed by filtration or centrifugation. The heated solution was next brought to 0.5 saturation with $(NH_4)_2SO_4$ and the precipitate was carried thrice more through the precipitation with 0.2 to 0.4 saturated $(NH_4)_2SO_4$, as given above in step (5).

7. *NaCl Fractionation*—The final $(NH_4)_2SO_4$ precipitate was dissolved in 60 cc. of water and then dialyzed until salt-free. Any precipitate that appeared on dialysis was removed by centrifugation before the next step. The dialyzed solution was brought to pH 3.0 with 1.0 M HCl and made 0.06 saturated with NaCl. The precipitate was centrifuged off and the concentration of NaCl in the supernatant increased to 0.25 saturation by adding more saturated NaCl solution. This procedure may be repeated until the 0.25 saturated NaCl supernatant contains a constant nitrogen content after two successive precipitations with 0.25 saturated NaCl. Usually only one precipitation was found necessary to obtain the adrenocorticotropic hormone in pure form.

TABLE I

Yield in Each Step in Isolation Procedure with 50 Gm. of Acid-Acetone Powder As Starting Material

Isolation step No (see text)	Fraction	Yield, N content
		mg.
2	Na_2HPO_4 extract	1564
3	Dialyzed, soluble material of $(NH_4)_2SO_4$ ppt.	654
3	0.06 saturated NaCl supernatant	306
4	Dialyzed, soluble material of acetone-NH_3 ppt.	191
6	Heat-soluble material from $(NH_4)_2SO_4$ ppt.	109
7	Final NaCl ppt. (adrenocorticotropic hormone)	32

The method has been shown from more than twenty experiments to give consistent results in yield and biological activity. Table I summarizes the average yield in terms of nitrogen in each step of the isolation procedure. It may be seen that from 1 kilo of frozen sheep glands approximately 70 mg. of the hormone could be isolated.

Homogeneity Studies

Solubility—The solvent used was a barbital buffer (pH 3.0) containing 1.395 M NaCl. Equilibrium was achieved by constant rotation in a cold room at 3°. The results shown in Fig. 1 indicate that the preparation gives a solubility curve indicative of a single component. It may be added that no difference in biological activity was found in the soluble and insoluble material in samples containing 0.86 mg. of nitrogen per cc.

Electrophoresis—Electrophoretic studies, with the moving boundary method of Tiselius, were made extensively in the course of the isolation procedure. The preparation obtained in each step was examined for its electrophoretic behavior as to homogeneity and mobility. It was found that the dialyzed, soluble material of the $(NH_4)_2SO_4$ precipitate from the Na_2HPO_4 extract of acid-acetone powder (see step (3) of the isolation procedure) contained five electrophoretically distinct components, which are indicated in Fig. 2. As the purification proceeded, the number of components revealed in electrophoresis decreased. Fig. 2, *B* is a typical pattern obtained from the fraction before heat treatment (see step (5) of the

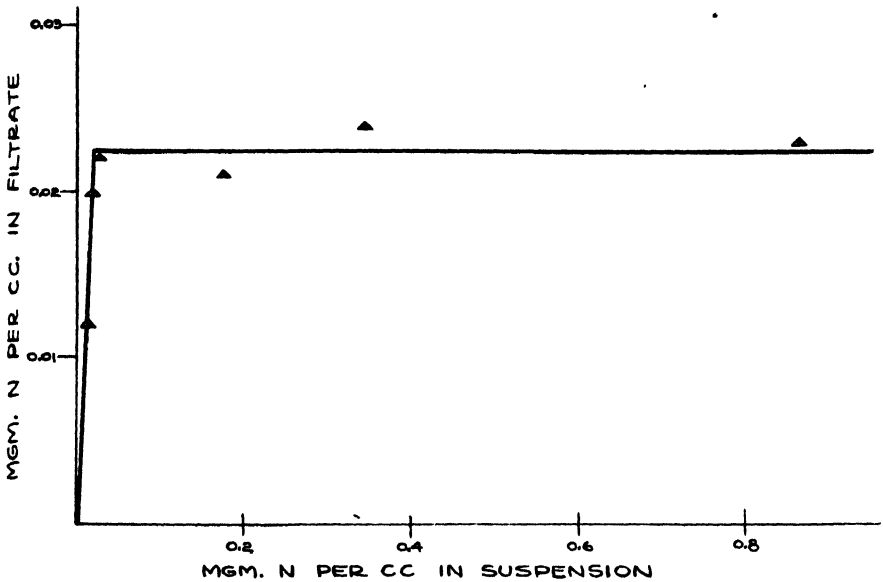


FIG. 1. Solubility of adrenocorticotropic hormone in the presence of increasing quantities of solid phase.

isolation procedure). Two components only were seen; the main component (fast moving) had a mobility of 4.8×10^{-5}, whereas the slow moving one had a mobility of 3.5×10^{-5} in phosphate buffer of ionic strength 0.10. These components were partially separated in the electrophoretic experiments and it was found that the fast moving component had practically all of the adrenocorticotropic activity. After the heat treatment, and the $(NH_4)_2SO_4$ and the NaCl fractionations, the slow moving component disappeared as shown in Fig. 2, *C*.

The adrenocorticotropic hormone has been examined electrophoretically in seven pH buffers; each experiment indicated that the material is a homo-

geneous substance. Typical patterns[3] taken at pH 2.20, 3.15, and 7.00 are presented in Fig. 2, *C*. Table II summarizes the mobility data at different pH values. Fig. 3 shows that the isoelectric point of the hormone is between pH 4.65 and 4.70.

Ultracentrifugation – A sample of the adrenocorticotropic hormone was examined in the laboratories of Professor J. W. Williams for its ultra-

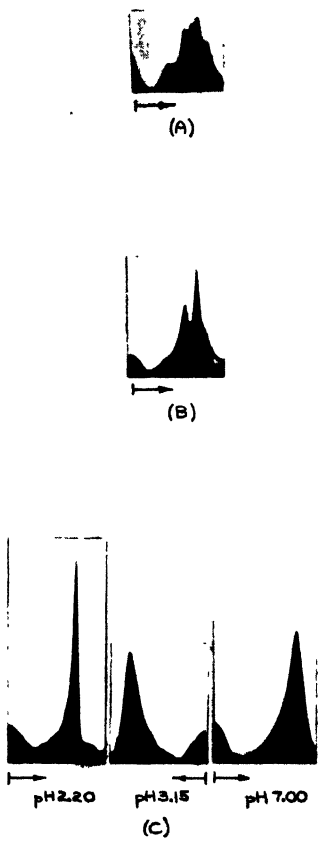


FIG. 2. Electrophoresis patterns of the ascending boundaries for (*A*) the phosphate extract of the acid-acetone powder, (*B*) the fraction before the steps with $(NH_4)_2SO_4$ at pH 7.00, and (*C*) the pure adrenocorticotropic hormone at pH 2.2, 3.15, and 7.00.

centrifugal behavior. In a private communication, Professor Williams has written as follows: "We find for S_{20} the value 2.1×10^{-13} cm. per second per dyne, and for D_{20} the value 10.5×10^{-7} sq. cm. per second. The

[3] It may be noted that the base-lines of these patterns are irregular owing to the use of a poor schlieren lens. For the distorted base-line see (13).

combination of sedimentation and diffusion constants gives a molecular weight of 20,000 if we assume the protein has a partial specific volume of 0.75, as is usually done. The actual scale line-distance curves for the hormone indicate very nicely that the substance must be homogeneous or very nearly so." We are greatly indebted to Professor Williams and his colleagues for these determinations.

TABLE II

Electrophoretic Mobility of Adrenocorticotropic Hormone in Buffers of Different pH, of Ionic Strength 0.1, at 1.5°

pH	Buffer	μ (10^5)
7.00	Phosphate	−5.0
6.78	"	−4.6
5.84	"	−3.5
4.50	Acetate	+1.0
4.10	"	+4.10
3.15	Barbital	+6 4
2.20	Chloride	+8.6

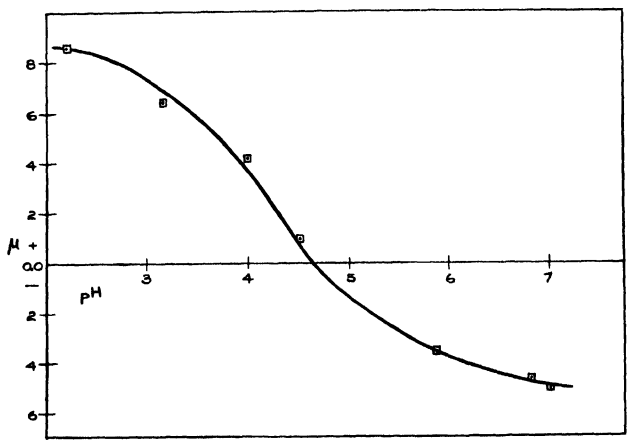


FIG. 3. Electrophoretic mobility of adrenocorticotropic hormone as a function of pH.

Biological Test—For the study of biological properties of a hormone, it is important to ascertain its freedom from other biologically active contaminants. By our present physicochemical methods appreciably less than 1.0 per cent impurity in protein preparations cannot be detected. On the other hand, by sensitive bioassays contaminants present in as little as 0.1 per cent are detected. In the claim for purity of a preparation, such

contaminations cannot be overlooked, even though they have not altered its chief physicochemical characteristics.

When the hormone in its final form was assayed in hypophysectomized male and female rats, it was found that it contained less than 0.10 per cent of the follicle-stimulating, the interstitial cell-stimulating, and the thyrotropic hormones. The adrenocorticotropic hormone inhibits the growth of rats and is antagonistic to the growth hormone in hypophysectomized rats;[4] such experiments and those conducted with adrenalectomized rats show that the hormone is freed from the growth hormone. Data for urinary nitrogen to be reported by us subsequently are strikingly confirmatory of this conclusion.

In the acid-acetone powder, the main hormonal contaminant is the lactogenic hormone. An important achievement by the method herein described is the complete removal of lactogenic hormone from adrenocorticotropic preparations. When 20 mg. of the hormone were injected subcutaneously into month-old squabs for a period of 4 days (3), no crop sac reaction occurred, indicating its freedom from lactogenic activity.

When the method of Burn (14) and a standard preparation of pressor principle (pitressin, Parke, Davis and Company) were used, it was found[5] that the adrenocorticotropic hormone contains 0.04 I.U. per mg. Since the most potent pressor preparation has 200 I.U. per mg. (15), it may be stated that the pressor contamination in the adrenocorticotropic hormone is less than 0.02 per cent.

Biological Assay

The importance of a sensitive assay method for the isolation of a hormone hardly needs to be emphasized. Among the difficulties experienced by others in securing highly potent adrenocorticotropic extracts may be reckoned the lack of a satisfactory method of assay. The methods of Moon (9) and of Bates *et al.* (10) were not extremely sensitive. The use of hypophysectomized rats in assay of the hormone have allowed the reliable detection of small quantities of hormone. This test animal can be used satisfactorily either to maintain or repair the adrenal cortex after atrophy. A detailed account of these methods will be given in a separate paper but a brief description must be presented here.

Adrenal Repair—The method, based on repair of the adrenals of hypophysectomized rats, is as follows: Female rats are hypophysectomized at 26 to 28 days of age; 14 days later the rats are injected intraperitoneally once daily for 4 days, followed by autopsy examination 96 hours after the first injection. The minimum amount of the hormone which gives micro-

[4] Unpublished data of this laboratory.

[5] We are indebted to Dr. Muriel Boelter for these determinations.

scopically recognizable beginnings in the repair of the adrenals in such rats is about 0.02 mg.

Adrenal Maintenance—The maintenance test used is as follows: Male rats, 40 days of age, are hypophysectomized and injected daily (except Sunday) from the day of operation for 15 days (thirteen injections). The adrenal weight of uninjected hypophysectomized animals regresses during

TABLE III

Bioassay of Adrenocorticotropic Hormone in 40 Day-Old Hypophysectomized Male Rats (Maintenance Test)

Daily dose	No. of rats	Body weight at autopsy	Adrenal weight	Thymus weight
mg.		*gm.*	*mg.*	*mg.*
1.0	10	107.8	40.1 ± 0.8*	46.8
0.20	15	117.4	25.1 ± 0.9*	130.8
0.10	15	117.9	22.3 ± 1.0	193.7
0.05	25	118.6	19.5 ± 1.0	190.8
0.02	14	107.8	15.6 ± 0.7	201.2
0.00	19	127.5	12.0 ± 0.5	222.4

* The ± values are the standard deviations.

TABLE IV

Effect of Adrenocorticotropic Hormone on Hypertrophy of Adrenals in Normal Male Rats

Six rats were used for each experiment.

Age of rats	Total dose*	Body weight			Adrenals		Thymus weight
		Onset	Autopsy	Gain or loss	Observed weight	Weight per 100 gm. body weight	
days	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg*	*mg.*
89	15.0	302	286	−16	75.8	26.5	79.0
89	0.0	301	336	35	39.0	11.6	296.0
26	30.0	59	132	73	85.3	64.6	41.9
26	0.0	59	202	143	30.3	15.0	455.5

* 1 mg. daily was divided into three intraperitoneal injections at 8 hour periods; on Saturday, two injections were given and on Sunday, one injection.

this period from about 26 mg. to a constant weight of 12 mg. The amount of hormone which maintains the adrenal at 26 mg. is about a 0.2 mg. daily dose. Table III shows the effect on adrenal weights of injection of daily doses ranging from 0.02 to 1.0 mg.

The ability of the pure adrenocorticotropic hormone to cause hypertrophy of the adrenals in normal male rats is shown in Table IV.[6] It may be noted

[6] The effect of the hormone on the body weight and the thymus will be discussed in a later paper.

that in 26 day-old rats the adrenal size increased 400 per cent in a period of 30 days injection.

Since the effect of the hormone is greatly increased by several instead of a single injection in 24 hours, it is necessary to use the same assay method in estimating the potency of each fraction secured in the isolation procedure. We may state that approximately 10 mg. daily of the acid-acetone powder are needed to maintain the adrenal at 26 mg. in the maintenance test, whereas only 0.20 mg. of the pure hormone is necessary.

Some Chemical Characteristics

The elementary analysis of a pure sample shows it to contain 46.35 per cent carbon, 5.89 per cent hydrogen, 15.65 per cent nitrogen, and 2.3 per cent sulfur. There is no carbohydrate, phosphorus, or cysteine in the

TABLE V

Effect of Trichloroacetic Acid on Activity of Adrenocorticotropic Hormone (4 Day Adrenal Repair Test)*

Three rats were used for each experiment.

Solutions	Total dose	Adrenal repair
	mg.	
Treated hormone	0.50	1 +, 2 +, 2 +
	0.10	−, −, −
	0.05	−, −, −
Untreated hormone	0.50	3 +, 3 +, 2 +
	0.10	1 +, 2 +, 2 +
	0.05	2 +, 2 +, 1 +

* The adrenal weights were not significantly increased.

hormone. It is very soluble in water and is only partly precipitated at its isoelectric point (pH 4.6 to 4.7).

In 2.5 per cent trichloroacetic acid solution, the hormone is 85 to 90 per cent precipitated. The trichloroacetic acid precipitate after being dissolved in a solution of pH 7.5 had lost its biological activity. As shown in Table V, the treated hormone contains only about one-tenth of the adrenocorticotropic activity. This characteristic differs from that observed with the lactogenic and interstitial cell-stimulating hormones (4).

Stability

No change in the biological activity of the hormone was observed after treatment in 10 per cent ammonia solution for 2 days at room temperature. When the hormone solution (0.5 per cent protein in buffer of pH 7.5 and ionic strength 0.10) was immersed in boiling water, its activity remained

the same. The stability of the hormone toward heat is extraordinary for a protein solution. It may be recalled that both the lactogenic and interstitial cell-stimulating hormones are destroyed when subjected to the temperature of boiling water for 1 hour (2, 16).

When a 0.2 per cent solution of the hormone in 0.10 M NaOH was heated to 100° for 30 minutes, the adrenocorticotropic activity was almost completely destroyed but when the hormone was dissolved in 0.1 M HCl and kept at boiling temperature for 60 minutes the activity was not appreciably altered. These results are summarized in Table VI.

TABLE VI

Effect of Heat on Activity of Adrenocorticotropic Hormone in 0.10 M HCl and 0.10 M NaOH (4 Day Adrenal Repair Test)

Three rats were used for each experiment.

Solvent	Length of treatment	Total dose	Adrenal histology
	min.	*mg.*	
0.1 M HCl	30	1.0	2+, 2+, 2+
		0.5	2+, 2+, 2+
		0.2	1+, 2+, 2+
		0.1	1+, 2+, 2+
	60	1.0	2+, 2+, 2+
		0.5	2+, 2+, 2+
		0.2	1+, 1+, 1+
		0.1	1+, 1+, 1+
0.10 M NaOH	30	2.0	−, −, −
		1.0	−, −, −
		0.5	−, −, −
		0.1	−, −, −

Effect of Trypsin and Pepsin

The solvent used for the pepsin experiments was 0.1 M HCl and that for the trypsin, phosphate buffer (pH 7.5). The hormonal concentration was always 0.40 per cent, while the enzyme concentration was 0.1 to 0.5 mg. per cc. Both the trypsin and pepsin employed were commercial preparations, pepsin (Lilly) and trypsin (Pfanstiehl). The temperature of incubation was 37° ± 1°.

The extent of digestion was determined from the non-protein nitrogen, trichloroacetic acid being used as the precipitant. The procedure used was essentially that of Northrop (17). To stop the digestion, the solutions were put into a boiling water bath for 15 minutes and then cooled immediately. After proper dilution had been made, bioassays were made in either the hypophysectomized male (maintenance test) or female (repair test) rats.

Table VII gives the results of treatment of the hormone with trypsin. It may be noted that the adrenocorticotropic activity was destroyed when 26 per cent of the protein had been digested by trypsin at the end of 15 minutes. However, no significant diminution of its activity was found when 18 per cent of the hormone had been hydrolyzed.

TABLE VII

Effect of Trypsin on Adrenocorticotropic Activity (15 Day Maintenance Test)*

Time of incubation	Concentration of enzyme	Protein digested	Daily dose	No. of rats	Adrenal weight
min.	*mg.*	*per cent*	*mg.*		*mg.*
15	0.1	18	0.20	5	23.0
			1.00	5	31.3
15	0.2	26	0.20	5	15.0
			1.00	3	16.3
120	0.4	41	0.10	4	15.2
			1.00	5	14.0

* For the control weights of the adrenals see Table III.

TABLE VIII

Effect of Crude Pepsin on Adrenocorticotropic Activity

Time of incubation	Concentration of enzyme	Percentage of original protein digested	Maintenance test*			Repair test		
			Daily dose	No. of rats	Adrenal weight	Total dose	No. of rats	Adrenal repair
min.	*mg. per cc.*		*mg.*		*mg.*	*mg.*		
40	0.5	37				1.00	3	3 +, 3 +, 2 +
						0.20	3	3 +, 2 +, 2 +
						0.05	3	1 +, 2 +, 1 +
						0.025	3	1 +, 1 +, 1 +
120	0.1	36	1.00	5	34.2			
			0.50	4	33.2			
			0.10	5	22.6			
			0.03	5	21.0			

* For the control weights of the adrenals see Table III.

The effects of pepsin on the potency of the hormone are shown in Table VIII. The data (obtained from both assay methods) indicate that the adrenocorticotropic activity remained unchanged even when a considerable part of the protein (36 to 37 per cent) had disappeared as a result of peptic digestion.

The interpretation of such results will be deferred, as further experiments with crystalline enzyme preparations are in progress.

SUMMARY

A method has been devised for the isolation of pure adrenocorticotropic hormone. From electrophoretic, sedimentation, and solubility experiments, the preparation answers essential criteria for belief that it is a single substance. Biological tests indicate that the hormone is freed from the other anterior hypophyseal hormones.

The molecular weight of the adrenocorticotropic hormone is approximately 20,000 and the isoelectric point about 4.7.

The hormone is very stable at 100° in buffer of pH 7.5 and in 0.1 M HCl solutions, but not in 0.1 M NaOH. Its adrenocorticotropic activity is destroyed by trichloroacetic acid and by tryptic digestion, but it has a marked stability to peptic digestion (unimpaired when 37 per cent digested).

BIBLIOGRAPHY

1. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Biol. Chem.*, **140**, 43 (1941).
2. White, A., Bonsnes, R. W., and Long, C. N. H., *J. Biol. Chem.*, **143**, 447 (1942).
3. Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **146**, 627 (1942).
4. Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, **92**, 355 (1940); *Endocrinology*, **27**, 803 (1940); *J. Am. Chem. Soc.*, **64**, 367 (1942).
5. Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B., and Chow, B. G., *Science*, **92**, 188 (1940).
6. Smith, P. E., *Am. J. Anat.*, **45**, 205 (1930).
7. Collip, J. B., Anderson, E. M., and Thomson, D. L., *Lancet*, **2**, 347 (1933).
8. Lyons, W. R., *Proc. Soc. Exp. Biol. and Med.*, **35**, 645 (1937).
9. Moon, H. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 649 (1937).
10. Bates, R. W., Riddle, O., and Miller, R. A., *Endocrinology*, **27**, 781 (1940).
11. Noble, R. L., and Collip, J. B., *Endocrinology*, **29**, 934 (1941).
12. Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, **96**, 450 (1942).
13. Li, C. H., and Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, **64**, 1586 (1942).
14. Burn, J. H., *Quart. J. Pharm. and Pharmacol.*, **4**, 57 (1931).
15. Irving, G. W., Jr., and du Vigneaud, V., *Ann. New York Acad. Sc.*, **43**, 273 (1943).
16. Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, **30**, 969 (1942).
17. Northrop, J. H., *J. Gen. Physiol.*, **16**, 41 (1932).

PREPARATION AND PROPERTIES OF PITUITARY ADRENOTROPIC HORMONE*

By GEORGE SAYERS,† ABRAHAM WHITE, AND C. N. H. LONG

(From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven)

(Received for publication, May 27, 1943)

The isolation of a pituitary hormone which specifically stimulates the adrenal cortex is of particular interest at a time when the physiological importance of this endocrine gland has been fully recognized. Relatively little attention has been given to the chemical purification of pituitary adrenotropic hormone. Two groups of investigators (1, 2) have prepared purified fractions which have been shown to stimulate the adrenals of the hypophysectomized rat. The exact physiological potency and the degree of purification of these fractions are difficult to assess, although one preparation was reported (3) to have at least one other type of pituitary activity; namely, lactogenic.

In preliminary communications, two groups of investigators (4, 5) have reported the isolation of pure adrenotropic hormone from pituitary glands. The two methods of preparation are distinctly different; in one salt fractionation is employed (4), in the other isoelectric precipitation (5).

This paper presents in detail the method developed (5) for the isolation of adrenotropic hormone from pituitary tissue and describes some of its properties.

Isolation of Adrenotropic Hormone

Fresh, whole, hog pituitary glands have usually been employed in the fractionation studies.[1] The fractionation steps described are for 1 kilo of tissue.

Step 1—"Crude prolactin" is prepared as described by Lyons (2) with the modification suggested by White, Bonsnes, and Long (7). Yield, 5 gm.

* This investigation has been aided by grants from the Fluid Research Fund of Yale University School of Medicine, the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association, and the Committee on Research in Endocrinology, National Research Council.

† The data in this paper are taken from a dissertation presented by George Sayers as partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University.

[1] Studies have also been conducted starting with fresh, whole, sheep and beef pituitary glands. However, purification of fractions from the glands of these two species has not as yet been carried to completion. The higher concentration of adrenotropic hormone in hog pituitaries (6) led to the temporary concentration of efforts on this source of hormone.

Step 2—The "crude prolactin" is dissolved in 50 ml. of water at pH 9.0 with the aid of 2 N NaOH and the pH lowered to 8.0 by the careful addition of 0.1 N HCl. The precipitate which forms is separated by centrifugation and discarded. The supernatant is diluted with water to a volume of 235 ml. and precipitates appearing on careful adjustment of the pH to 6.6 and 5.4 (by addition of 0.1 N HCl) are removed by centrifugation and discarded. To the supernatant (at pH 5.4) are added 17 ml. of saturated $(NH_4)_2SO_4$. A small amount of precipitate which forms overnight in the ice box is centrifuged and discarded. 4 volumes of acetone are added to the supernatant and the mixture placed in the ice box overnight. The precipitate is centrifuged. This is Fraction M. Yield, 1.9 gm.

Step 3—Fraction M is dissolved in 133 ml. of water with the aid of 0.1 N NaOH and the solution mixed with one-half its volume of concentrated NH_4OH. The ammoniacal solution is allowed to stand at room temperature for 7 hours, and 1800 ml. of acetone added. The precipitate (Fraction N) is recovered at the centrifuge. Yield, 1.5 gm.

Step 4—Fraction N is dissolved in 75 ml. of water and dialyzed against repeated changes of distilled water until free of inorganic salt. Any precipitate which forms during dialysis is discarded. The pH of the solution is carefully adjusted with 0.1 N HCl to 5.4 and centrifuged free of precipitate. Lowering the pH of the supernatant to 4.7 precipitates adrenotropic hormone. It is washed three times with acetone at the centrifuge and dried *in vacuo* over concentrated sulfuric acid. This is Fraction A. Yield, 400 mg.

Some adrenotropic activity may be recovered from the supernatant of the precipitate at pH 4.7 by the addition of acetone to a concentration of 40 per cent. Yield of precipitate (Fraction B), 350 mg.

Exposure to concentrated ammonium hydroxide for 7 hours (Step 3) is not sufficient to destroy pressor principles completely. Preparations of adrenotropic hormone, previously exposed to ammonia, have been assayed for pressor activity in the cat.[2] Assays of antidiuretic activity (8) have also been conducted. The data show that 1 mg. of adrenotropic hormone exhibited a pressor and an antidiuretic activity equivalent to 0.6 unit of pitressin (Parke, Davis and Company). This amount of contamination of posterior lobe principle is indeed very small, inasmuch as pressor substances having an activity of 200 units per mg. have been isolated (9). It has been possible to reduce the degree of activity of posterior lobe principle in adrenotropic hormone preparations by extending the time of exposure to concentrated NH_4OH (Step 3) to 20 hours from the usual 7 hours. A preparation of the hormone exposed to this longer treatment with ammonia

[2] Grateful acknowledgement is made to Dr. L. S. Goodman of the Department of Pharmacology for the pressor assay in the cat.

showed no measurable loss in adrenotropic activity, while antidiuretic activity was reduced. 1 mg. of this hormone preparation produced an antidiuretic response equivalent to 0.1 unit of pitressin.

Physical and Chemical Properties of Adrenotropic Hormone

Qualitative Tests—Fraction A, the purified adrenotropic hormone, gives the following positive color reactions, biuret, Hopkins-Cole, and Millon's. The Molisch reaction is negative. The labile sulfur test is positive, but the nitroprusside reaction for free sulfhydryl groups is negative. The hormone gives the nitroprusside reaction after treatment in solution with sodium cyanide.

The adrenotropic hormone is readily precipitated from dilute solution by 20 per cent sulfosalicylic acid, by 20 per cent trichloroacetic acid, and by 5 per cent lead acetate solution. It is apparent that the adrenotropic hormone behaves as a typical protein.

Elemental Analysis—The purified hormone has been subjected to elemental analysis.[3] The analyses show 0.4 per cent ash and the following elementary composition (expressed on an ash- and moisture-free basis), carbon 50.64, hydrogen 6.23, nitrogen 15.47, and sulfur 2.33 per cent.

Electrophoretic Studies of Adrenotropic Hormone—Electrophoresis of purified adrenotropic hormone, Fraction A, has been conducted in the Tiselius apparatus. The apparatus used in this laboratory is equipped with the Philpot scanning device. Five electrophoretic experiments have been conducted, two at pH 7.95, the others at pH 3.26, 4.13, and 6.37. At all pH values studied, the initial protein boundary migrated as a single component. Fig. 1 shows the electrophoretic patterns obtained in two of the electrophoresis experiments at pH values most removed from and on either side of the isoelectric point.

The electrophoretic mobilities calculated from the data obtained in the Tiselius experiments are plotted against pH in Fig. 2. The isoelectric point of adrenotropic hormone is at pH 4.7 to 4.8. The value of du/d pH (change in mobility with pH) for the hormone is 1.5×10^{-4} sq. cm. per second per volt.

Ultracentrifugation of Adrenotropic Hormone—Sedimentation studies have been conducted in a Beams analytical ultracentrifuge arranged for optical study in the visible region by the Toepler schlieren method, as developed for the ultracentrifuge by Philpot (10). A 2 per cent solution of the hormone in 1 per cent NaCl at pH 7.5 was employed. The initial protein peak present at the start of the experiment sedimented at a uniform rate and gave no evidence of a second component during the period of ultracentrifugation. Values of 2.04, 2.11, and 2.04 were obtained for the sedi-

[3] Conducted by Mr. J. F. Alicino.

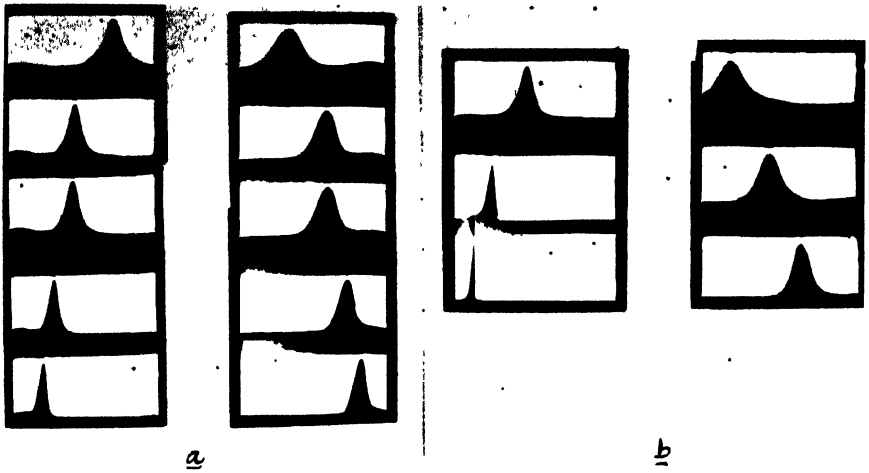


FIG. 1. Electrophoretic patterns of adrenotropic hormone (Fraction A). (*a*) protein concentration, 1.0 per cent; phosphate buffer in 0.1 M NaCl; ionic strength, 0.3; pH 7.95; temperature, 4°. Photographs of the ascending boundary (left) after 120, 185, 281, 295, and 477 minutes (reading from bottom to top) and of the descending boundary (right) after 118, 186, 284, 287, and 480 minutes. (*b*) protein concentration, 0.8 per cent; acetate buffer in 0.1 M NaCl; ionic strength, 0.2; pH 3.26; temperature, 3°. Photographs of the ascending boundary (left) after 0, 80, and 220 minutes and of the descending boundary (right) after 179, 309, and 469 minutes.

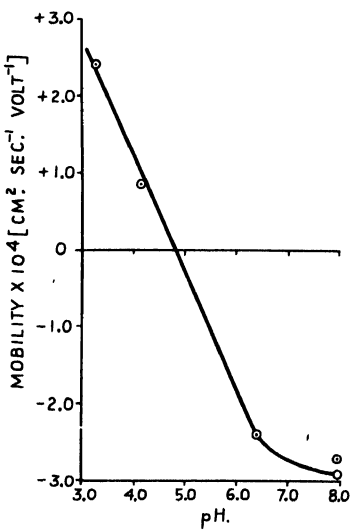


FIG. 2. Electrophoretic mobility of adrenotropic hormone (Fraction A)

mentation constant in three consecutive runs. Fig. 3 shows a typical series of photographs obtained in one of these experiments.

In the absence of data for the diffusion constant, the sedimentation constant obtained indicates the protein to have a molecular weight of approximately 20,000.

Methods of Assay

Three methods have been employed for the assay of the adrenotropic hormone activity of various pituitary preparations.

Adrenal Hypertrophy of Intact Immature Rat—The procedure is essentially that described by Moon (3), with several modifications.

21 day-old male rats were used. A solution of the material to be assayed is injected intraperitoneally three times daily for 3 days, the volume of each injection being 0.25 to 0.50 ml. 16 hours after the last injection the animals are killed with illuminating gas, and the adrenals dissected carefully and weighed to the nearest tenth of a mg. by means of a torsion balance.

A unit of adrenotropic activity is defined as the total amount of material which, when injected as described, will produce a 50 per cent increase in

FIG. 3. Sedimentation of adrenotropic hormone (Fraction A); photographs by the Philpot schlieren method at 40, 100, and 160 minutes, reading from left to right. Speed 39,000 R.P.M. (110,000 × *g*).

adrenal weight over that of control animals injected with water. Adrenal weight is expressed as mg. per 100 gm. of body weight at the beginning of the injection period. This precludes the possibility that any change in body weight, occurring during hormone treatment, may influence the results.

Assay of Adrenotropic Hormone in Hypophysectomized Rat—Purified adrenotropic hormone preparations have been assayed on the basis of their ability (*a*) to repair the atrophied adrenals, and (*b*) to maintain the adrenals of the hypophysectomized rat.

Repair of Adrenals of Hypophysectomized Rat—Male rats[4] approximately 45 days of age are hypophysectomized and, beginning on the 10th day after hypophysectomy, the material to be assayed is injected intraperitoneally three times daily for 3 days. 16 hours after the last injection the animals are killed with illuminating gas and the adrenals, testes, thyroid, and thymus weighed accurately on a torsion balance.

Maintenance of Adrenals of Hypophysectomized Rat—Male rats[5] approximately 45 days of age are hypophysectomized and, beginning on the 1st

[4] From the Breeding and Laboratory Institute, Brooklyn.

[5] Sprague-Dawley strain.

day after hypophysectomy, are injected intraperitoneally with the material to be assayed once daily for 14 days. 24 hours after the last injection they are killed with illuminating gas and the adrenals, testes, thyroid, and thymus weighed on a torsion balance.

Assay Data

Assay of Fractions in 21 Day-Old Rats—The results of the assay of various fractions in the 21 day-old rat are given in Table I. Desiccated, whole, hog pituitary powder serves as a reference standard from which to judge the degree of purification of adrenotropic hormone. However, a considerable portion of the activity of pituitary tissue as measured by the increase in size of the adrenals of the intact immature rat may be due to non-adrenotropic factors. From data to be disscussed, it will be evident that posterior lobe principles are fraction contaminants that con-

TABLE I

Assay of Adrenotropic Hormone Preparations in 21 Day-Old Rat

Preparation	No of rats used	Mg equivalent to 1 unit
Desiccated whole hog pituitaries	19	35
Adrenotropic preparation from hog pituitaries*	20	5
Adrenotropic preparation from sheep pituitaries*	15	5
Fraction M (beef)	10	5
Fractions M and N (hog)	21	5
Fraction A (hog)	19	5

* Made according to Li, Simpson, and Evans (4).

tribute to the hypertrophy of the adrenals of this animal. Thyrotropic hormone, by stimulating the thyroid, would also contribute to this hypertrophy (11). Caution, therefore, must be exercised in judging absolute increases in potency of relatively pure fractions over the original standard powder. Approximately 35 mg. of the desiccated, whole, hog pituitary powder represent 1 adrenotropic unit.

Purified adrenotropic hormone made according to the directions of Li, Simpson, and Evans (4) is approximately 7 times more potent than desiccated pituitary tissue when assayed in the 21 day-old rat. From Table I it can be seen that there is no difference between preparations made from hog and from sheep pituitaries. 1 adrenotropic unit is represented by 5.0 mg. of the Li, Simpson, and Evans preparation.

Fractions M, N, and A do not differ in their ability to cause adrenal hypertrophy. 1 unit of adrenotropic hormone is represented by 5.0 mg. of each of these fractions; this is a potency equal to that of the preparation

described by Li, Simpson, and Evans (4) and claimed by these investigators to be the pure adrenotropic hormone. Fractions M and N differ from one another only in that the latter is much less contaminated with posterior lobe principles.

There is no evidence of a species difference in adrenotropic activity when comparisons are made among fractions obtained from beef, sheep, and hog pituitary tissue. The absence of an apparent increase in potency by further fractionation of Fraction N in order to isolate the final adrenotropic product, Fraction A, must mean that the impurities in Fraction N are already so small in quantity as to be non-detectable by this assay method. It may be noted here that assays in the hypophysectomized rat, to be discussed later, suggest that preparations of Fraction N have some growth hormone activity.

It should be emphasized that assay of adrenotropic hormone in the 21 day-old rat is not rigidly specific. Adrenal stimulation by factors other than adrenotropic hormone, *e.g.* posterior lobe principles, is possible because of the presence of the animal's pituitary. The assay is technically simple, requires a relatively short time, and has been very useful in the present study as a guide in the fractionation of adrenotropic hormone.

Results of Assay of Adrenotropic Hormone Preparations in Hypophysectomized Rat. Repair of Adrenals—Fractions N, A, and B have been assayed by the method based upon the repair of the atrophied adrenals of the hypophysectomized rat. The data are shown in Table II. Fractions N and B exhibit growth-promoting activity. The changes in body growth suggest that growth hormone is not precipitated at pH 4.7, but remains in the supernatant solution; a purification has therefore been attained in preparing Fraction A from Fraction N. This purification is not clearly evident as an increase in adrenotropic activity, since Fractions N and A both produce the same amount of adrenal hypertrophy. It may be added that no significant changes were seen in the weight of the thymus, thyroid, or testes of any of the treated animals with the possible exception of a slight increase in the weight of the testes of animals administered Fraction B.

From the results it is evident that adequate amounts of preparations of potent adrenotropic hormone can repair the atrophied adrenals of the hypophysectomized rat in the short space of 3 days.

Maintenance of Adrenals—Assay results in the hypophysectomized rat by the maintenance method are shown in Table III.

The weights of the adrenals of the hypophysectomized rats given Fraction A, the purified adrenotropic hormone, in doses of 100, 50, 25, 12.5, 10, and 5 γ per day are significantly greater than those of hypophysectomized controls. At a daily dose level of 2 γ the weight of the adrenals is not significantly greater than the control values. Actual hypertrophy, *i.e.* in-

crease in adrenal weight above that of the glands of normal intact controls, was definite at a dose level of 100 γ per day in the Brooklyn animals but not in the Sprague-Dawley rats. Evidence of hypersecretion in this latter strain was seen from the beginning thymic involution at this dose level of hormone. The better response of the Brooklyn rats may be accounted for by the relatively greater dose they receive per unit of body weight. These animals at the age of 45 days weigh about 35 per cent less than Sprague-Dawley animals of the same age.

There appears to be a wide difference between the daily dosage of hormone which prevents adrenal atrophy and that necessary to produce actual

TABLE II

Assay of Adrenotropic Hormone Activity in Hypophysectomized Rat by Repair Method

Preparation	Total dose	No. of rats	Change in body weight*	*P* values†	Paired adrenal weight per 100 gm. body weight*	*P* values;‡ comparison between injected animals and	
						Hypophysectomized controls	Non-hypophysectomized controls
	mg		*gm.*		*mg.*		
Non-hypophysectomized controls		9			17.3 ± 0.9		
Hypophysectomized controls...		7	−2 ± 1.7		9.6 ± 0.7		
Fraction N....	5	5	+3 ± 0.4	0.02	18.6 ± 2.3	<0.01	0.6
" A... ..	5	4	−4 ± 2.9	0.5	18.3 ± 2.0	<0.01	0.6
" B	5	3	+5 ± 1.2	0.02	14.2 ± 1.4	<0.01	0.1

* Means and standard errors.

† *P* values for body weight; calculated for small series (12). Comparison between hypophysectomized controls and injected animals.

‡ *P* values for adrenal weight.

adrenal hypertrophy. This range extends from approximately 12.5 to 50 γ. This must mean that only a small amount of hormone is required to maintain the adrenals, while a relatively large amount is necessary to produce hypertrophy. The minimum effective daily dose for maintenance of adrenal weight in the hypophysectomized rat is 25 γ. Although the results suggest that the daily injection of 12.5 γ may actually be adequate under the conditions of assay, the data are as yet too few to warrant describing the hormone as definitely effective at this dose level.

Fraction B is less potent than Fraction A in maintaining the adrenals of the hypophysectomized rat. From Table III, Fraction B may be estimated to be about one-half as potent as Fraction A. This agrees with the experimental results obtained in the repair method of assay (Table II).

In view of the presence of traces of posterior lobe principles in fractions of purified adrenotropic hormone discussed previously, two posterior lobe preparations, pitressin (Parke, Davis and Company) and pars neuralis hormone (13),[6] were assayed by this method. These substances do not prevent the atrophy of the adrenal cortex of the hypophysectomized rat (Table III). From Table III it can also be seen that purified prolactin

TABLE III

Assay of Adrenotropic Hormone Activity in Hypophysectomized Rat by Maintenance Method

Preparation	Daily dose	No. of rats	Paired adrenal weight per 100 gm. body weight *	*P* values;† comparison between injected animals and	
				Hypophysectomized controls	Non-hypophysectomized controls
	γ		mg.		
Non-hypophysectomized controls		11	17 1 ± 0 9		
Hypophysectomized controls		8	10.4 ± 0.5		
Fraction A	100	7	18.8 ± 0.7	<0.01	0.2
" "	100	5‡	24.7 ± 1 3	<0.01	<0.01
" "	50	6	16.4 ± 0 6	<0 01	0.6
" "	50	2‡	20.7 ± 2.8	<0 01	0.2
" "	25	4	17.5 ± 2.0	<0.01	0.9
" "	12.5	3	17.0 ± 0.8	<0 01	1.0
" "	10	3	14.7 ± 0.8	<0.01	0.2
" "	5	5	14.2 ± 0.9	<0.01	0 1
" "	2	4	11.5 ± 0.8	0.3	<0.01
" B	100	7	15.7 ± 0.6	<0.01	0.3
Pars neuralis hormone.	100	2	11 7 ± 1.1	0 3	0.05
Pitressin (Parke, Davis).	1.0 I.U.	5	11.5 ± 0.7	0.2	<0.01
Prolactin	25	5	10.4 ± 0.5	1.0	<0.01

* Means and standard errors.

† *P* values for adrenal weight; calculated for small series (12).

‡ From the Breeding and Laboratory Institute, Brooklyn.

did not prevent adrenal atrophy. This method of assay appears to be specific for adrenotropic hormone.

The body weights (not included in Table III) of the injected animals showed no increase in any of the experiments, indicating the absence of growth hormone activity in the preparations at the dose levels employed. No alterations in weight of the thyroid or testes were seen in the injected

[6] Kindly supplied by Dr. H. B. van Dyke.

animals. Moreover, when assayed for lactogenic hormone in the pigeon, preparations of highly active adrenotropic hormone failed to produce a positive crop gland response in a total dose of 10 mg. 0.1 mg. of purified prolactin stimulates a response under similar conditions.

DISCUSSION

Metabolic studies with crude extracts have created considerable confusion regarding the exact number of hormones produced by the anterior pituitary. Repetition of these studies with pure hormones will be expected to clarify existing knowledge of pituitary physiology. The physiological approach to an accurate understanding of the anterior pituitary is contingent upon the availability of as many as possible of the gland secretions in a chemically pure form. It will indeed be striking to study the metabolism of the hypophysectomized animal treated with one or a combination of the isolated and purified pituitary hormones.

The following hormones of the anterior pituitary may now be considered to have been isolated in a form which satisfies the accepted criteria of homogeneity, prolactin (7, 14, 15), luteinizing hormone (16), and adrenotropic hormone (4, 5). In addition, reports indicate that a high degree of purity has been attained for the thyrotropic hormone (17) and for the growth hormone (18). Claims for the existence of additional "metabolic" hormones will require reexamination in the light of data to be obtained with the purified preparations of adrenotropic, thyrotropic, lactogenic, and growth hormones available at present.

Although the pure adrenotropic preparation which has been examined in some detail in the present study has been obtained from hog pituitary glands, highly purified fractions of adrenotropic hormone have also been obtained from bovine and sheep pituitaries. Preliminary studies of these preparations suggest that they are similar to, if not identical with, the product obtained from hog glands. This observation has been supported by personal conversations with Dr. C. H. Li who has stated that the pure adrenotropic hormone prepared from sheep glands (4) has many properties identical with those reported here for the hormone obtained from hog pituitaries. These properties include biological activity, nitrogen content, isoelectric point, and sedimentation constant. It may be noted that the isoelectric point found for adrenotropic hormone is considerably different from that of pH 6.6 to 6.8, formerly assumed to be correct (2).

Although the assay data for adrenotropic hormone are based upon alterations in adrenal size, it has also been established in this laboratory that the hormone stimulates the functional activity of the adrenal cortex. This activity is manifested by changes in cholesterol concentration of the adrenals of rats injected with adrenotropic hormone (19) and in the amount

and distribution of lipid in the adrenal cortices of these animals.[7] In fact, 25 γ of pure adrenotropic hormone administered daily to the hypophysectomized rat are sufficient to maintain not only normal adrenal size but also the normal concentration of cholesterol and lipid in the adrenals.

SUMMARY

1. A method has been described for the preparation of pure adrenotropic hormone from pituitary tissue. The product has the properties of a protein.

2. The hormone preparation fulfils two criteria for protein purity; it behaves as a single component in the Tiselius apparatus and in the ultracentrifuge.

3. The isoelectric point of adrenotropic hormone, as determined by electrophoresis, is between pH 4.7 and 4.8. The sedimentation constant of the hormone, $S_{20} = 2.04$ to 2.11, indicates the molecular weight to be approximately 20,000.

4. Under the conditions of assay used, a total dose of 5 mg. of the hormone is adequate for the complete restoration of adrenal weight in the rat 10 days after hypophysectomy. A daily dose of 25 γ of the hormone completely maintains size and function of the adrenals of the hypophysectomized rat when injections are begun the 1st day after hypophysectomy. 5 γ daily produce a definitely measurable adrenal-stimulating effect, while a daily dose of 2 γ fails to elicit a detectable response.

5. Under the conditions of bioassay employed, the adrenotropic hormone preparation exhibits none of the following anterior pituitary hormonal activities, growth, gonadotropic, thyrotropic, or lactogenic.

BIBLIOGRAPHY

1. Collip, J. B., Anderson, E. M., and Thomson, D. L., *Lancet*, **2**, 347 (1933).
2. Lyons, W. R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **5**, 198 (1937).
3. Moon, H. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 649 (1937).
4. Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, **96**, 450 (1942).
5. Sayers, G., White, A., and Long, C. N. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 199 (1943).
6. Astwood, E. B., and Tyslowitz, R., *Federation Proc.*, **1**, pt. 2, 4 (1942).
7. White, A., Bonsnes, R. W., and Long, C. N. H., *J. Biol. Chem.*, **143**, 447 (1942).
8. Gilman, A., and Goodman, L. S., *J. Physiol.*, **90**, 113 (1937).
9. Irving, G. W., and du Vigneaud, V., *Ann. New York Acad. Sc.*, **43**, 273 (1943).
10. Philpot, J. St. L., *Nature*, **141**, 283 (1938).
11. Cohen, R. S., *Am. J. Anat.*, **58**, 143 (1935).
12. Fisher, R. A., Statistical methods for research workers, Edinburgh, 4th edition (1932).

[7] Unpublished data.

13. van Dyke, H. B., Chow, B. F., Greep, R. O., and Rothen, A., *J. Pharmacol. and Exp. Therap.*, **74**, 190 (1942).
14. White, A., Catchpole, H. R., and Long, C. N. H., *Science*, **86**, 82 (1937).
15. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Gen. Physiol.*, **23**, 433 (1940).
16. Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B., and Chow, B. F., *Science*, **92**, 178 (1940).
17. Ciereszko, L. S., and White, A., *Federation Proc.*, **1**, pt. 2, 105 (1942).
18. Marx, W., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **147**, 77 (1943).
19. Sayers, G., Sayers, M. A., White, A., and Long, C. N. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 200 (1943).

SPECTROPHOTOMETRIC STUDIES

XI. THE DIRECT MICRO SPECTROPHOTOMETRIC DETERMINATION OF CYTOCHROME C*

By OTTO ROSENTHAL and DAVID L. DRABKIN

(From the Harrison Department of Surgical Research and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, May 13, 1943)

We have undertaken to secure more extensive information on the distribution and concentration of cytochrome *c* in normal and neoplastic mammalian tissues.[1] A prerequisite was the development of a relatively simple procedure for the quantitative determination of the pigment in small amounts of material, such as those obtainable from the organs of rats or from the surgical clinic. The method, which will be described, consists of two main steps. The cytochrome *c* is isolated from the tissue samples, and obtained in solution free from interfering pigments such as hemoglobin. It is then determined as ferrocytochrome *c* (the pigment reduced with $Na_2S_2O_4$) by means of direct spectrophotometry.

The isolation of the pigment is accomplished by material modifications in and simplification of the procedure of Fujita, Hata, Numata, and Ajisaka (2). In agreement with the findings of Potter and DuBois (3) the original method of the Japanese workers has been found unnecessarily tedious and unreliable with reference to the completeness of recovery of cytochrome *c*. The point of principal weakness in the method proved to be the use of successive precipitations by means of acetone, which does not accomplish the desired concentration of the pigment and is productive of losses. Precipitation with trichloroacetic acid has been substituted, with resulting satisfactory recovery of added cytochrome *c*.

Spectrophotometry is carried out by means of a new adaptation in technique which permits the analysis of a small volume of solution contained in a deep cuvette of capillary bore. By this micro spectrophotometric means ferrocytochrome *c* can be determined in appreciably smaller amounts of tissue than hitherto possible when direct methods of analysis were utilized. The method is applicable to tissue samples varying in wet (fresh) weight from 0.2 to 20 gm., depending upon their cytochrome *c* content which varies in the corresponding order of approximately 300 γ per gm. to

* The expenses of this work were defrayed in part by grants from the Penn Mutual Life Insurance Company Foundation for the Study of Neoplastic Diseases and from the George de Benneville Keim Fund.

[1] Preliminary reports have appeared (1).

1 to 2 γ per gm. The method possesses advantages of simplicity and directness over procedures which involve the preparation of fresh enzyme extracts for each set of determinations (3, 4), and which require indirect methods of analysis (determination before and after reduction).

Analytical Procedures

Collection of Tissues—The organs of freshly killed animals or human organs or tissues after their surgical removal were collected in ice-cooled jars and stored in a refrigerator until used. Extraction was usually performed within 6 hours of the collection of the tissue. When extraction could not be carried out upon the same day, the jars were kept in the freezing compartment overnight. Analyses indicated that storage for several days in the frozen state did not alter the amount of cytochrome *c* recovered. This circumstance is useful, since other analyses performed upon aliquots of the same tissue,[2] had to be carried out promptly, and occasionally required brief postponement of the cytochrome *c* isolation.

Isolation of Cytochrome c—The procedure, which is the outcome of testing several variations in technique, may be conveniently presented as consisting of three steps. A full description is provided, since certain tissues were found to require minor modifications in treatment appropriate to themselves, and complete recovery of the cytochrome *c* depends upon faithful attention to details.

1. *Extraction of Tissues*—The tissues are minced finely with scissors and the mince thoroughly mixed. Mucous tissues are first rinsed with Ringer's solution and drained on filter paper to remove surface mucus. Brain is mashed and mixed by means of a stainless steel micro spatula. After withdrawal of samples for the determination of dry weight and for other analytical procedures,[2] a weighed amount of the mince is ground in a mortar with powdered Pyrex glass. When the amount of mince does not exceed 1 gm., the weighing is most conveniently carried out on a microscopic cover-glass, which is then ground together with the mince.

The mince is thoroughly mixed and ground successively with the following per gm. of mince: 0.25 ml. of distilled water, 1.25 ml. of 0.5 M H_2SO_4, and 0.5 ml. of 2 M NH_4OH. By these means a fluid mixture is obtained, and its total volume is taken as 3 times the weight of the mince. The mixture is allowed to stand with occasional stirring in the covered mortar for a period of 15 minutes. This is the usual extraction procedure, and is essentially that employed by Fujita *et al.* (2). However, as will be pointed out, occasionally greater tissue dilutions are required. The extract is now centrifuged for 15 minutes at 4500 R.P.M. in a Swedish angle centrifuge, yielding a usually somewhat turbid supernatant fluid. The latter

[2] Rosenthal, O., and Drabkin, D. L., unpublished data, manuscript in preparation.

is transferred to a graduated cylinder, and the volume measured and recorded. The volume recovered approximates 60 to 70 per cent of that of the original mixture, and is therefore considered to represent an equivalent percentage of the original tissue weight.

2. *Removal of Hemoglobin*—To the extract in the graduated cylinder is added an equal volume of an $(NH_4)_2SO_4$ solution, containing 0.5 gm. of the salt per ml. The cylinder is stoppered, and immersed in a water bath at 56° for a period of 15 minutes. Under these conditions the cytochrome *c* and some spectroscopically inert proteins remain completely soluble, while the flocculation of other proteins including hemoglobin starts immediately and progresses during the period of heating. As a rule a bulky, reddish top layer and a more compact bottom sediment separate. Although the precipitate may be removed promptly by filtration through a Whatman No. 40 filter after the cylinder is cooled, it is safer to refrigerate the mixture for several hours or, preferably, overnight before filtration. This insures a complete separation of precipitate and mother liquor. Certain tissues, such as the salivary glands, yield especially bulky precipitates. In such cases filtration is preceded by centrifugation. Omission of centrifuging results in extremely slow filtration, which leads to losses in cytochrome *c* presumably due to the adsorption of the pigment on the clogged filter. Centrifuging alone is insufficient, since the lighter particles are not completely removed. At this stage if desirable the filtrate may be kept without deterioration for several days in the refrigerator.

3. *Precipitation of Cytochrome c*—The whole filtrate or a suitable aliquot thereof is transferred to a graduated "heavy duty" type of centrifuge tube.[3] 1 ml. of 90 per cent trichloroacetic acid is added per 10 ml. of filtrate (3), and the solution mixed with a stirring rod and refrigerated for a period of 30 minutes. Longer refrigeration is permissible. Ordinarily flocculation occurs within 30 minutes after the addition of the acid, and the mother liquor appears clear. If the latter remains turbid, sedimentation is probably incomplete. Under such circumstances 0.5 gm. of $(NH_4)_2SO_4$ per 10 ml. of initial filtrate is added, and the addition of this quantity of salt is repeated until complete precipitation of the cytochrome *c*-containing material is accomplished. An unnecessary excess of salt should be avoided, since in solutions of high density the precipitate will rise to the surface and its separation by centrifuging will be delayed.

When the precipitation appears to be complete, the stirrer is rinsed with

[3] For high speed centrifuging (3500 to 4500 R.P.M.) in an angle centrifuge, ignition tubes (Pyrex, 16 × 125 mm. and 18 × 150 mm., with a capacity respectively of 12 and 22 ml.) have been found excellent. Such tubes after calibration have been used by us for all operations which did not involve measurement of the collected filtrate and supernatants.

saturated $(NH_4)_2SO_4$ solution and the tube centrifuged at 3500 to 4500 R.P.M. for 30 minutes. The supernatant fluid is now separated into another centrifuge tube and checked for turbidity. After decantation of the supernatant fluid the tubes (containing the precipitate) are inverted upon filter paper and allowed to drain. The sediment is then brought into solution by stirring with 1 M NaOH, added drop by drop from a semimicro burette. Usually 0.25 ml. of the base is required.[4] A small amount of insoluble material (probably denatured protein) remains. The solution is brought to a suitable volume of the order of 1 to 1.5 ml. by the addition of distilled water, or with sufficient 1 M HCl to make the final concentration in OH^- approximately 0.2 M. After removal of the insoluble material by a brief centrifugation, the solution is ready for spectrophotometry.

In tissues low in cytochrome *c* which require the extraction of 5 gm. or more of tissue, the volume of filtrate obtained is greater than 15 ml. Under such conditions the precipitation is accomplished in several centrifuge tubes. The sediments are dissolved as above, quantitatively combined, and brought to a total volume of the order of 3 to 5 ml. Insoluble matter is removed by centrifuging and the volume of supernatant fluid measured. The solution is acidified to pH approximately 4 with 2.5 M and 0.1 M HCl, with hexamethoxy red[5] as the indicator. (If interruption of analysis is desired for convenience, the acidified solution may be stored in the refrigerator for several days.) The cytochrome *c* is then reprecipitated by the addition of trichloroacetic acid as described above. By this means the cytochrome *c* which is recovered from as much as 20 gm. of fresh tissue can be concentrated to a volume of 1 ml.

Micro Spectrophotometric Determination of Ferrocytochrome c—The Bausch and Lomb polarization spectrophotometer (6) has been adapted to the analysis of small volumes of solution in a technique which may be designated as micro spectrophotometry. The usual Bausch and Lomb cuvette of 1 cm. depth employed for hemoglobin studies has an internal diameter of 1.7 cm. and a capacity of 2.3 ml. This has been replaced by a cuvette of 0.47 cm. capillary bore and 3.4 cm. depth, whose capacity is 0.59 ml. It is obvious that only one-fourth the volume of solution required for the 1 cm. chamber is sufficient to fill the special 3.4 cm. cuvette. The factors of capacity and depth operate together to increase sensitivity ap-

[4] In the rare instances in which some turbidity remains in the decanted supernatant fluid, it is treated with $(NH_4)_2SO_4$ and centrifuged. The sediment is dissolved in 1 M NaOH, and this solution is used for dissolving the main sediment.

[5] Hexamethoxy red, 2,4,2′,4′,2″,4″-hexamethoxytriphenylcarbinol (5), is convenient in this operation, since it is unique in changing from colorless to red on the acid side of pH 4.6. The indicator was kindly supplied by Dr. H. Lund. A 0.1 per cent solution in alcohol was employed. Since spectrophotometry is carried out upon alkaline solutions, the presence of the indicator offers no interference.

proximately 14-fold (4 × 3.4). Thus, solutions may be kept low in volume, thereby permitting the preparation of more concentrated extracts from small amounts of tissue, and the small volumes may be examined in a relatively deep layer.

The entrance pupils of the polarization photometer (König-Martens type) of the Bausch and Lomb instrument have a diameter of 0.8 cm., nearly 2 times that of the capillary cuvette. The adaptation of the cuvette to the photometer was accomplished by the use of an iris diaphragm as a

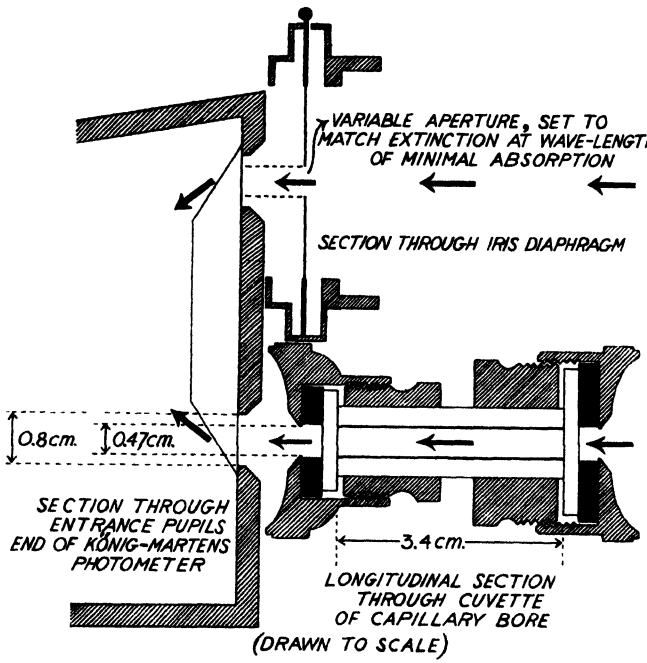


FIG. 1. An exact diagrammatic representation of the alignment of the photometer, capillary cuvette, and iris diaphragm

balancing device in front of one of the entrance pupils. The diaphragm and cuvette are aligned between the lenses which collimate the parallel beams of light and the photometer, as close to the latter as possible. The arrangement is shown in Fig. 1, a longitudinal section drawn exactly to scale. The diaphragm serves as a variable aperture, and is set to match extinction at the wave-length of minimal absorption for the particular pigment studied. In the case of ferrocytochrome *c* this adjustment is made at a wave-length of 630 mμ at which clear solutions of the pigment in the range of concentrations used have very little absorption (7). The photometer is set to read 43.0° (45.0° corresponding to 100 per cent trans-

mission), and the iris aperture is then adjusted so that the two optical fields match. This procedure effectively balances out inequalities of optical aperture in the two pathways of light, and to some extent also corrects for turbidity. After coincident setting at a wave-length of 630 mμ, spectrophotometry is carried out in the usual manner, with a spectral interval of 1.5 to 2.0 mμ, at wave-lengths of 550 mμ (maximum of the α-band), 535 mμ (minimum between the α- and β-bands), and 520 mμ (maximum of the β-band). In the calculation of concentration the extinctions or optical densities ($D = -$ log transmission) at only wave-lengths of 550 and 535 mμ and the ratios of the two extinction values to each other are employed. The absorption constants, ϵ (c = 1 mM per liter, d = 1 cm.), used are at these wave-lengths respectively 29.28 and 9.45, with their ratio equal to 3.10. These values have been obtained under the conditions of measurement which apply in the capillary cuvette for a 0.2 M NaOH solution of a purified preparation of ferrocytochrome *c*, whose constants have been established previously upon an iron basis, with the usual 1 cm. cuvette (7). The molecular weight magnitude of reference is 13,000, referred to pure cytochrome *c* with an iron content of 0.43 per cent (8).

The calibration of cuvette depth was carried out by direct comparison of extinction values obtained upon a solution of oxyhemoglobin in the new cuvette and in one of established 1 cm. depth. The reduction of cytochrome *c* to its ferro form was accomplished by means of solid dithionite, $Na_2S_2O_4$, approximately 0.1 mg. being added per ml. of solution. The reduced solution was transferred to the capillary cuvette by means of a medicine dropper with the tip drawn out into a fine capillary. Bubbles were avoided by placing the filled capillary dropper deeply in the cuvette, and withdrawing slowly during the act of emptying.

Comments

Extraction of Cytochrome c—When amounts of tissue smaller than 2 gm. were used, or with viscous tissues such as salivary glands or brain, it was found that larger volumes of extracting agents than those recommended by Fujita *et al.* (2) are necessary to insure recovery of a sufficient aliquot of the extract. In these instances the extracting agents—water, 0.5 M H_2SO_4, and 2 M NH_4OH (step (1), under analytical procedures)—were increased proportionately so that their ratios to the total volume of mixture remained unaltered. Under such conditions only one alteration was encountered. The pH of the more dilute extracts approaches 1.65 in comparison with approximately 2.5 in the more general procedure. This change in pH is not critical. An examination of Table I indicates that the degree of dilution of the mince is without influence upon the results in the case of all tissues listed except perhaps liver. Duplicate analyses are in

general within the range of error (±5 per cent) of measurement in the graduated glassware used in the analytical procedure (Experiments 1 and 6, Table I).

Precipitation of Pigment—In our method of precipitating cytochrome *c* from the filtrates trichloroacetic acid is added to a final concentration of 8 per cent as in the method of Potter and DuBois (3). The extracts, prepared under our conditions, already contain approximately 25 per cent of $(NH_4)_2SO_4$. Even this concentration of salt is insufficient in some cases for complete precipitation, so that more $(NH_4)_2SO_4$ must be added (Experiment 5, Table II). Potter and DuBois (3) have reported that cytochrome

TABLE I

Reproducibility of Analyses under Various Conditions of Dilution of Tissue Mince

Experiment No.	Tissue	Species	Sample*	Dilution of mince	Cytochrome *c*	(B − A/A) × 100
					γ per gm. dry weight	*per cent*
1	Liver	Rat	A	1:3.15	477	+4
			B	1:3.20	496	
2	"	Rabbit	A	1:3.03	201	−24
			B	1:6.00	153	
3	Submaxillary glands	Rat	A	1:3.17	396	+11
			B	1:6.04	439	
4	Brain cortex	Rabbit	A	1:3.02	304	+1
			B	1:6.00	308	
5	Kidney cortex	Pig	A	1:3.10	259	+9
			B	1:5.95	282	
6	" "	Man	A	1:3.00	35.4	−1
			B	1:3.50	35.1	

* Samples A and B are from the same mince.

c may be precipitated quantitatively from their dilute preparations of the pigment by the addition of trichloroacetic acid alone. This apparent discrepancy appears to be explainable by the fact that our filtrates are prepared differently. They may be freer of non-cytochrome *c* proteins than the filtrates employed by the Wisconsin investigators.[6] This was suggested by unsuccessful attempts to precipitate quantitatively cytochrome *c* by the addition of trichloroacetic acid alone to solutions of the purified pig-

[6] In our method of preparing filtrates, hemoglobin, certain other proteins, and fats are removed in a single step at acid pH when $(NH_4)_2SO_4$ is added to a concentration of approximately 25 per cent. In the method of Potter and DuBois (3) in most cases hemoglobin is not removed, although certain other proteins are removed at neutral reaction.

ment, free of $(NH_4)_2SO_4$. Complete precipitation could be accomplished, however, by means of both trichloroacetic acid and $(NH_4)_2SO_4$ (Experiment

TABLE II

Recovery of Added Cytochrome c under Different Conditions

Experiment No.	Tissue	Material to which cytochrome *c* is added	No. of pptns. with trichloroacetic acid	Calculated concentration of total cytochrome *c* before		Additional $(NH_4)_2SO_4$ required	Calculated final concentration* of		Recovery of total cytochrome *c*
				1st pptn.	2nd pptn.		Added cytochrome *c*	Total cytochrome *c*	
				γ per ml.†	*γ per ml.*	*gm. per ml.*	*γ per ml.*	*γ per ml.*	*per cent*
1	‡	$(NH_4)_2SO_4$, 25%	1	19.3		0.35§	128.5	128.5	101
2	‡	$(NH_4)_2SO_4$, 25%, + diastase	1	19.3		0.35	128.5	128.5	94
3	Liver (rabbit)	Filtrate‖ + diastase	1	17.8		0.20	137.0	142.7	88
4	" (rat)	Original extract¶	1	48.4			66.4	161.0	98
5	Oxalated blood (dog)	" "	1	20.4		0.15	127.5	127.5	94
6	Kidney cortex (rabbit)	" "	1	19.3			67.9	128.8	105
7a**	Submaxillary gland (rat)	Mince	1	34.4			54.0	105.2	99
7b**	" "	"	1	21.0			74.0	139.5	89
8	Kidney cortex (man)	"	2	8.4	24.3	0.25††	29.2	34.9	105
9	Rectal carcinoma (man)	"	2	5.46	38.2	0.20††	110.8	122.1	97
10	Lung (rat)	"	2	7.44	41.4	0.15††	94.9	112.6	81

* The concentration of the solution used for spectrophotometry.

† In 1:3 initial dilutions of the mince, 1 ml. of filtrate is equivalent to 0.167 gm of fresh tissue.

‡ Solutions of purified cytochrome *c*.

§ No precipitation occurred before the further addition of $(NH_4)_2SO_4$.

‖ Turbid filtrate.

¶ Clear extract.

** In Experiment 7a the mince was diluted 1:3.02, in Experiment 7b 1:6.0.

†† Added only in the second precipitation with trichloroacetic acid.

1, Table II). It is probable that in filtrates containing "inert" protein, the pigment may be adsorbed on the protein, and thereby more readily removed by means of trichloroacetic acid.

Two filtrate constituents, glucoprotein and glycogen, encountered respectively in filtrates from gastric mucosa and liver, interfere with the quantitative determination of cytochrome *c*. The presence of the gastric glucoprotein resulted in final solutions of such turbidity that determination was impossible, and no chemical means has been found to separate the glucoprotein from any cytochrome *c* which may be present. In the case of glycogen, when it was present in amounts producing turbidity, enzymatic decomposition of the polysaccharide by means of clarase, a commercial preparation of pancreatic diastase, proved feasible. In such instances after clarification of neutralized filtrates by enzymatic digestion at 37°, they were acidified and cytochrome *c* precipitated in the usual way. Experiments 2 and 3, Table II, illustrate the recovery of cytochrome *c* under such conditions. Some loss of the pigment may occur, owing to the diastase treatment, but it is considered to be of negligible dimensions for the problem at hand.

The other experiments (Nos. 4 and 6 to 10) in Table II show the recovery of cytochrome *c*, added at different stages of the analytical procedure. Although it is recognized that recovery of added cytochrome *c* cannot be considered adequate as proof of completeness of recovery of the pigment present in the original tissue, nevertheless such experiments are useful in judging losses of cytochrome *c* which may occur in certain steps of the procedure. The recovery experiments also justify the method of calculation of the cytochrome *c* content of the tissue mince in which it is assumed that the filtrate recovered represents a true aliquot. The recovery of added cytochrome *c* in all tissues except lung (Experiment 10, Table II) appears very satisfactory. Occasional figures above 100 per cent are no doubt due to the error of measurement of volume in graduated glassware.

Spectrophotometry of Sample, and Calculations Employed—Table III gives the data employed in the spectrophotometric calibration of the depth of the new capillary cuvette. The figures in the fifth column of this table ($D_2 - D_3$) suggest that the main function of the variable aperture diaphragm is to compensate for the base-line absorption (approximately equal non-specific absorption over the range of wave-lengths used) due to the constricted aperture of the capillary cuvette in comparison with the larger aperture of the entrance pupils of the photometer. The technique of coincident extinction setting at the wave-length of minimal absorption also compensates to some extent for turbidity. Most of the filtrates obtained by our method are not optically clear. Full correction for base-line absorption due to turbidity (9) or yellowish pigments (possibly flavines) occasionally present is accomplished by the method of calculation employed. The ratio of extinction or optical density at a wave-length of 550 mμ (maximum of the α-band of ferrocytochrome *c*) to that at 535 mμ (minimum between the α- and β-bands) provides the correction factor.

Base-line absorption increases extinction at both these wave-lengths by an equal quantity, resulting in a diminution of the ratio of the two extinction values. The corrected extinction value at a wave-length of 550 mμ is obtained from the observed value as follows:

$$D^{\lambda\,550}_{\text{corrected}} = D^{\lambda\,550}_{\text{observed}} \cdot \left(\frac{1 - \frac{1}{r_{\text{observed}}}}{1 - \frac{1}{r_{\text{theory}}}} \right) \qquad (1)$$

TABLE III

Calibration of Depth of Capillary Cuvette with Oxyhemoglobin Solution of Known Concentration

Wave-length	D_1,* 1 cm cuvette	D_2,* capillary cuvette, uncompensated†	D_3,* capillary cuvette, compensated‡	$D_2 - D_3$	Depth of capillary cuvette
mμ					cm.
630		0.740§	0.122§	0.618	
578	1.226‖	1.971§	1.383§	0.588	3.38¶
562	0.695‖	1.423§	0.787§	0.636	3.40¶
542	1.194‖	1.971§	1.360§	0.611	3.42¶
Average.					3.40

* $D = -\log T$, transmission, as a fraction of unity.

† Iris diaphragm not employed.

‡ Extinction at a wave-length of 630 mμ (minimal absorption) set arbitrarily at a low value, and match of the optical fields at this wave-length secured by adjustment of the iris diaphragm.

§ The oxyhemoglobin concentration is 0.0270 mM per liter (the solution used in the 1 cm. cuvette is diluted 1:3).

‖ The oxyhemoglobin concentration is 0.081 mM per liter, referred to a molecular weight equivalent of 16,700.

¶ The depth of the new cuvette in cm. was calculated according to the example, 1.383 × 3/1.226 = 3.38 cm.

where r_{observed} is the ratio $D(\lambda\,550)/D(\lambda\,535)$ obtained upon the filtrate and r_{theory} is 3.10, the same ratio for a purified, optically clear solution of ferrocytochrome *c* (Table IV). The concentration, *c*, of cytochrome *c* in mM per liter (referred to a molecular weight of 13,000) is now obtained by application of the equation,

$$\frac{D^{\lambda\,550}_{\text{corrected}}}{\epsilon_{\lambda\,550}} \times \frac{1}{3.4} = c \text{ (in mM per liter)} \qquad (2)$$

where ε is the absorption constant (extinction for a concentration of 1 mM per liter and a depth of 1 cm. (Table IV, Experiment 4)).

The concentration, c, may be obtained directly by a simplified expression derived from Equations 1 and 2.

$$c \text{ (in mM per liter or micromoles per ml.)} = \left(\frac{D^{\lambda\ 550}_{\text{observed}} - D^{\lambda\ 535}_{\text{observed}}}{\epsilon_{\lambda\ 550} - \epsilon_{\lambda\ 535}} \right) \times \frac{1}{3.4} \quad (3)$$

TABLE IV

Comparison of Absorption Constants, ϵ, for Optically Clear Solutions of Ferrocytochrome c at Different pH Levels in the 1 Cm. and in the Capillary Cuvette

ϵ is the extinction for a concentration of 1 mM per liter, referred to a molecular weight of 13,000, and a depth of 1 cm.

Experiment No.	Cuvette	pH*	Wave-length	ϵ	$\epsilon_{\lambda\ 550}/\epsilon_{\lambda\ 535}$
	cm.		mμ		
1	1	4.10	550 (α)†	26.11	
			535 (m)	7.59	3.44
			520 (β)	15.37	
2	1	13+	550 (α)	28.38	
			535 (m)	7.29	3.89
			520 (β)	14.73	
3	Capillary, 3.4	4.10	630	0.06‡	
			550 (α)	26.90	
			535 (m)	9.77	2.76
			520 (β)	17.97	
4	" 3.4	13+	630	0.06‡	
			550 (α)	29.28§	
			535 (m)	9.45§	3.10
			520 (β)	17.63	

* Determined by glass electrode. The value of 13+ indicates a pH greater than 13 for cytochrome c in 0.2 M NaOH solution.

† α, maximum of the α-band, β, maximum of the β-band, m, minimum, with a spectral interval of 1.5 to 2 mμ.

‡ Extinction arbitrarily adjusted for a low value by means of the iris diaphragm at a wave-length of minimal absorption for ferrocytochrome c.

§ Absorption constants for ferrocytochrome c used for the calculation of concentration in determinations upon alkaline solutions with the capillary cuvette.

When the values of ϵ in Table IV, Experiment 4, are used, Equation 3 becomes

$$c \text{ (in mM per liter or micromoles per ml.)} = \frac{D^{\lambda\ 550}_{\text{observed}} - D^{\lambda\ 535}_{\text{observed}}}{67.4} \quad (4)$$

or

$$c \text{ (in mg. per liter or micrograms per ml.)} = (D^{\lambda\ 550}_{\text{observed}} - D^{\lambda\ 535}_{\text{observed}}) \times 192.8 \quad (5)$$

By these simple, direct means adequate correction for extinction due to turbidity and related factors is obtained.

TABLE V

Analyses of Cytochrome c Content of Liver and Kidney Cortex of a Mixed Population of Albino Rats

Experiment No.	No. of rats per experiment*	Mean weight of rats	Liver		Kidney cortex cytochrome *c*
			Evident turbidity† of filtrate	Cytochrome *c*	
		gm.		*γ per gm. dry weight*	*γ per gm. dry weight*
1	1	320	None	666	1850
2	7	170	"	588	1580
3	2‡	223	"	588	1420
4	7	45	"	865	1315
5	1	289	Slight	815	
6	1	206	"	479	1310
7	1	240	"	487	
8	1	360	Strong	(264)	1305
9	2	52	"	(342)	1230
10a	1§	210	"	(293)	
10b			Clarified‖	371	
Mean, values in parentheses not included				607	1430
S.e.¶				±59.8	±81.9
(S.e./mean) × 100				±9.8	±5.7
Mean, calculated** from 10 analyses reported by Potter and DuBois (3)				235	875††
S.e.				±2.8	±27.1
(S.e./mean) × 100				±1.2	±3.1
Mean, calculated** from 10 analyses reported by Stotz (4)				195	1170
S.e.				±10.0	±38.2
(S.e./mean) × 100				±5.1	±3.3

* In experiments with pooled organs approximately 1 gm. of the liver and 0.5 gm. of the renal cortex were taken from each animal for the preparation of the respective minces.

† The relationship of turbidity of liver filtrates and glycogen content is discussed in the body of the paper.

‡ These animals were the parents of a litter used for Experiments 4 and 9.

§ Cane sugar was fed as an exclusive diet (to produce a very high liver glycogen content) for a period of 36 hours before sacrifice of the animal. Aliquots of the filtrate were used in Experiments 10a and 10b.

‖ Filtrate clarified by treatment with diastase.

¶ S.e., standard error, calculated from $\sqrt{\Sigma d^2/n(n-1)}$.

** The original values of Potter and DuBois (3) and Stotz (4) are expressed upon a wet (fresh) weight basis with the molecular weight of cytochrome c taken as 16,500 instead of 13,000 (8). To convert the values reported by these workers into the terms used by us, their figures for liver were multiplied by (13,000/16,500) × 3.31 and for kidney by (13,000/16,500) × 4.49. The factors 3.31 and 4.49 represent our mean values for the ratio of wet weight to dry weight in liver and kidney respectively.

TABLE V—*Concluded*

†† This value is for whole kidney. Based upon our analyses of kidney cortex and medulla, a rough estimate of the corresponding value for kidney cortex may be obtained by multiplying by the factor 1.2.

Since many applications of this new micro spectrophotometric technique no doubt may be found, attention should be called to its present limitations related to the optics of the system.[7] An examination of the figures given in Table IV discloses that somewhat different ϵ values are obtained for ferrocytochrome *c* in the capillary cuvette-diaphragm technique and in the standard technique with the 1 cm. cuvette. It should be emphasized that for the establishment of precise spectrophotometric constants only the values obtained by the standard technique should be employed.

In Table V the results of analyses upon two rat[8] tissues, liver and kidney cortex, are presented, as well as a comparison of our results with those of other workers (3, 4). These tissues were chosen for the contrast they afford. Both are relatively high in cytochrome *c*, but appreciably more consistent results are shown in the case of the kidney cortex than in the case of the liver. The latter tissue has been found by us to yield more variable results with reference to cytochrome *c* content than most other rat tissues examined. A comparison of the mean values, given in Table V, indicates furthermore that our results upon the kidney are of the same order of magnitude (when calculated upon a similar basis) as those reported by Potter and DuBois (3) and by Stotz (4). However, our analytical results upon the liver are significantly higher (approximately 3 times) than those reported by these investigators. Our experiments suggest that there is roughly an inverse relationship between the cytochrome *c* recovered and the glycogen content of the liver. A possible influence of diet upon the cytochrome *c* oxidase system in liver has been suggested previously (10). In a forthcoming publication, now in preparation, we will present the results of analyses of cytochrome *c* in various normal and neoplastic tissues of different species.

[7] A rather remarkable phenomenon is exhibited by the capillary cuvette. When visually turbid solutions which transmit light poorly in a 1 cm. layer are placed in the cuvette, they appear completely transparent and clear, though distinctly yellowish in color when viewed through the 3.4 cm. capillary. The explanation of this optical phenomenon is uncertain, since spectrophotometrically such solutions behave as if turbidity is still present, non-specific base-line absorption (discussed in the body of the paper) being evident from the extinction values obtained.

[8] Albino rats were used. The animals were purposely not selected as to stock, age, size, or sex, in order to represent a mixed population. This lack of selection with reference to age groups, etc., may be a factor in producing somewhat greater variability in the cytochrome *c* content of tissues from individual animals in our study.

SUMMARY

A method is described for the isolation of cytochrome *c*, free of hemoglobin, from small amounts of tissues (0.2 to 20 gm., fresh weight). The isolated pigment is concentrated to small volumes (1.0 to 1.5 ml.).

A micro spectrophotometric technique, applicable to less than 0.8 ml. of fluid, has been developed, in which a cuvette of capillary bore and 3.4 cm. depth (capacity of 0.59 ml.) and a compensating iris diaphragm are utilized. By these means ferrocytochrome *c* has been determined after isolation from appreciably smaller amounts of tissue than has hitherto been possible by direct methods of analysis.

A simple method is presented for applying corrections for non-specific light absorption due to turbidity or related factors.

A comparison of our analytical findings with those in the literature upon the cytochrome *c* content of rat kidney cortex and liver is given. Our results upon liver are significantly (3 times) higher than those previously reported. This finding has been discussed.

BIBLIOGRAPHY

1. Rosenthal, O., and Drabkin, D. L., *Am. J. Med. Sc.*, **204**, 914 (1942); *Federation Proc.*, **2**, 69 (1943).
2. Fujita, A., Hata, T., Numata, I., and Ajisaka, M., *Biochem. Z.*, **301**, 376 (1939).
3. Potter, V. R., and DuBois, K. P., *J. Biol. Chem.*, **142**, 417 (1942).
4. Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
5. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 81, 89 (1928). Kolthoff, I. M., *J. Am. Chem. Soc.*, **49**, 1218 (1927).
6. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **98**, 719 (1932); **112**, 51 (1935–36).
7. Drabkin, D. L., *J. Biol. Chem.*, **140**, 373 (1941); **146**, 605 (1942).
8. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1804 (1941).
9. Drabkin, D. L., and Singer, R. B., *J. Biol. Chem.*, **129**, 739 (1939). Drabkin, D. L., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. xxxiv (1941).
10. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 297 (1942).

THE VERATRINE ALKALOIDS

XX. FURTHER CORRELATIONS IN THE VERATRINE GROUP. THE RELATIONSHIP BETWEEN THE VERATRINE BASES AND SOLANIDINE

By LYMAN C. CRAIG and WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, June 10, 1943)

Recent studies on the veratrine bases (1, 2) have shown that all those thus far studied are members of a closely related group of substances which has also been found to include the potato base, solanidine (3), and therefore related *Solanum* bases. All possess formulations with 27 carbon atoms and, with the exception of jervine (and pseudojervine), are tertiary bases. This close relationship has been substantiated by the isolation of the characteristic basic degradation product, 2-ethyl-5-methylpyridine, from each on dehydrogenation. The individual formulations are shown in Table I.

The data obtained have indicated the hexacyclic character of all of these bases. From active hydrogen determinations, all of the oxygen atoms in these formulations must be contained in hydroxyl groups. Solanidine is known to contain a carbon to carbon double bond (4), and the unsaturation which we have found to be present in cevine by the production of dihydrocevines (5), in the light of our later work, must be regarded in the same category. Recently, protoverine (6) has also been found to yield dihydro derivatives. To complete the picture, the degree of unsaturation of other veratrine bases has been similarly studied.

In the case of germine, a difficulty was encountered, since all attempts to hydrogenate it with an active platinum oxide catalyst, either as the base dissolved in methanol or after acidification with acetic acid or with an active nickel catalyst, were unsuccessful. However, isogermine, as the free base dissolved in methanol, could be readily hydrogenated to *dihydroisogermine*. On the other hand, germine on treatment with sodium in butanol was reduced to an isomeric *dihydrogermine*. Active H determinations on these products have given results in agreement with the reduction of a carbon to carbon double bond and not with the opening of one of the tertiary N rings to give a secondary base. Both rubijervine and isorubijervine in methanol solution, which contained an excess of acetic acid, were converted respectively to *dihydrorubijervine* and *dihydroisorubijervine*. The hydrogenation of an olefinic linkage in the former was shown by its acylation to a *diacetyldihydrorubijervine*, which retained the original basic character.

It is thus seen that all of these bases, with the exception of jervine, have yielded data which permit the conclusion that they are singly unsaturated hexacyclic tertiary bases in which the tertiary N must be common to two of the rings. In the case of jervine (2, 7), sufficient data have been obtained to show its close relationship to the group. However, it remains in a special category, since it reacts as a secondary base and contains at least two conjugated double bonds which can be hydrogenated to a tetrahydrojervine. The derived formulation of jervine is $C_{27}H_{39}O_3N$, and if, as appears probable, it is pentacyclic, it must still contain two unsaturated linkages, which have not been detected as yet by hydrogenation. This is a question which will have to be investigated further.

The close analogy of the veratrine bases to solanidine is thus evident. Solanidine has been conclusively shown by Soltyz and Wallenfels (8) and Rochelmeyer (9) to be a sterol derivative, since it yields methylcyclo-

TABLE I

Formulations of Veratrine Bases

Cevine	$C_{27}H_{43}O_8N$
Germine	$C_{27}H_{43}O_8N$
Protoverine	$C_{27}H_{43}O_9N$
Jervine	$C_{27}H_{39}O_3N$
Rubijervine	$C_{27}H_{43}O_2N$
Isorubijervine	$C_{27}H_{43}O_2N$
Solanidine	$C_{27}H_{43}ON$
Solasodine	$C_{27}H_{43}O_2N$

pentenophenanthrene on selenium dehydrogenation. It also readily yields a sparingly soluble digitonide with digitonin. It was of interest, therefore, to check the latter property with the veratrine alkamines. As recorded in the experimental part, isorubijervine was found to form quite readily a sparingly soluble digitonide. Rubijervine also appeared to form a digitonide, but only after longer standing. This property proved to be absent in the case of jervine, as well as with germine, isogermine, cevine, and protoverine. The significance of the outcome of the test in the case of the latter, which are more highly hydroxylated alkamines, cannot be certain. However, the promptness of the precipitation of isorubijervine (crystallization of the digitonide began within 30 minutes) is strong evidence in this case of a 3-(β)-hydroxyl group contained on Ring A of a steroidal structure.

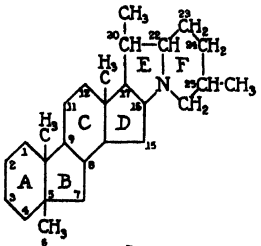
In certain ways, our experience with the veratrine alkamines has differed, at least from the recorded observations with solanidine and related *Solanum* bases. Thus we have not been able to isolate methylcyclopentenophenanthrene from the dehydrogenation mixture of any one of

the veratrine bases. Instead, from those which we have been able to study with sufficient thoroughness (except in the case of rubijervine), the more complicated hydrocarbons isolated behaved as tetracyclic or pentacyclic fluorene derivatives. We have, therefore, reinvestigated the neutral hydrocarbon fraction which results on dehydrogenation of solanidine, not only for confirmation of the recorded production of Diels' hydrocarbon, but also to determine whether any analogy to our experience with the veratrine bases could be found among the remaining dehydrogenation products. The dehydrogenation was carried out essentially as in the case of the veratrine bases, and Diels' hydrocarbon was isolated without difficulty. In addition, two other crystalline hydrocarbons were obtained which were identified as 2-methylphenanthrene and 1,2-dimethylphenanthrene. These substances were probably formed by the further degradation of Diels' hydrocarbon or its precursor. The most careful investigation, however, failed to reveal the presence of any fluorene hydrocarbons in the dehydrogenation mixture.

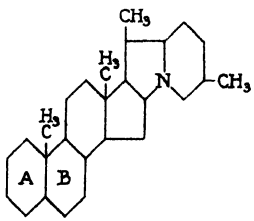
This difference of behavior taken alone might not be too conclusive, since, as recently reported, we had failed to obtain any evidence of the production of fluorene hydrocarbons among the dehydrogenation products from rubijervine (1). Instead, a hydrocarbon obtained as a major product appeared unquestionably to be a methylcyclopentenophenanthrene. This hydrocarbon did not prove to be Diels' hydrocarbon, but an isomer. The data obtained with the hydrocarbon and with its trinitrobenzene derivative agreed with those recorded for synthetic α-methyl-1,2-cyclopentenophenanthrene described by Ruzicka, Ehmann, Goldberg, and Hösli (10). But if such identity should be established, its formation would be difficult to interpret. The failure to obtain Diels' hydrocarbon from rubijervine, which is isomeric with the *Solanum* base, solasodine, from which Rochelmeyer (9) obtained this dehydrogenation product, thus requires explanation. But, irrespective of such differences, it is now clear that the whole veratrine group is closely related to the sterols, and any structural formula proposed, if not identical with the perhydro-1,2-cyclopentenophenanthrene structure of the latter, cannot depart greatly from it.

It would seem, therefore, on the basis of our present information, if correctly interpreted, that the possibility must still be seriously considered that Ring B contains 5 carbon atoms in those veratrine bases which yield fluorenes on dehydrogenation, as in Formula I, and perhaps 6 carbon atoms, as in Formula II, in those which yield phenanthrenes. If all should prove to be in the latter class, the fluorenes must result from a ring contraction during the dehydrogenation. Although ring enlargement from a 5-membered ring to a 6-membered ring, especially at high temperatures, has occasionally been observed, the reverse has not been noted as far as we are

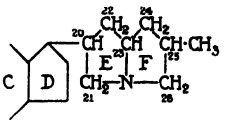
aware, at least under the usual conditions of dehydrogenation. We have naturally given consideration to a possibility which appears more difficult to reconcile with the data on hand; *i.e.*, that the fluorene character of the hydrocarbons obtained on dehydrogenation is due only to the recyclization of the side chain of Ring D on carbon atom 16, as generally accepted in the case of the pentacyclic naphthofluorenes which have been repeatedly encountered in the dehydrogenation of the sterols.



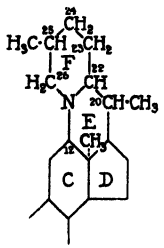
I



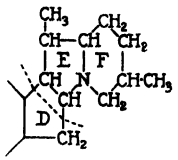
II



III



IV



V

Furthermore, there is still to be explained the origin of the unique hexanetetracarboxylic acid (and heptanetetracarboxylic acid) (11) which is formed during the chromic acid oxidation of cevine and more recently of germine (1). This hexanetetracarboxylic acid, the structure of which appears well established, is produced in excellent yield and, if correctly interpreted, indicates that two angular methyl groups are located on vicinal carbon atoms in these alkaloids unless it is again the result of a rearrangement. Formula II does not possess the necessary two angular methyl groups on vicinal carbon atoms. On the other hand, if rubijervine should have a 5-membered ring, as in Formula I, then the methylcyclopentenophenan-

threne obtained from it must be formed by ring enlargement during dehydrogenation. The nature of Ring B must, therefore, be regarded as a point which has not been established beyond question and will require further study.

The heterocyclic portion of Formula I or II is in complete accord with the sterol relationship, for it can be formed by condensation of the isooctyl side chain of the sterols with ammonia. This is somewhat analogous to the spiroacetal formula of the steroid sapogenins recently derived by Marker and Rohrmann (12), which involves double cyclization on C atom 22. However, another arrangement (Formula III), proposed by Clemo, Morgan, and Raper (13) for the heterocyclic portion of solanidine, is a pyrrolizidine which also can be formed from the side chain. The former, however, appears to be the arrangement which, while consistent with the sterol side chain, is more consistent with the production of β-picoline and 2-ethyl-5-methylpyridine as direct cleavage products. If the pyrrolizidine formula is correct, the 2-ethyl-5-methylpyridine formed in such good yield from solanidine would have to be the result of ring enlargement of Ring F to a 6-membered one, with rupture of the bond between carbon atoms 17 and 20. If such a rearrangement could occur, it would seem likely that a considerable amount of pyrrole formation could also take place. While we have not investigated the products from solanidine from this angle, we have very carefully investigated the volatile products from the dehydrogenation of a number of the veratrine alkaloids in search of pyrrole derivatives which might give direct evidence of the nature of Ring E, but in all cases have been unsuccessful. The pyrrole red test reported in earlier papers on solanidine does not appear to be significant, since it might arise from unsaturated nitrogen derivatives among the unstable degradation products. Finally, it should be pointed out that the formation of N-methyl-β-pipecoline (14) during the Zn dust distillation of cevine could be even less readily reconciled with a pyrrolizidine structure, since this would involve a rearrangement from a fully saturated, 5-membered nitrogen ring to a piperidine.

There is still another possibility which might be considered; *viz.*, cyclization on carbon atom 12, as shown in Formula IV. However, such an arrangement appears incompatible with the film measurements (15) and also with the interpretation of the unsaturated precursor of the dicyclic base, $C_{10}H_{19}N$, as a direct decomposition product from the soda lime distillation of cevine. The production of this base now appears best interpreted as the result of cleavage of Ring D, as in Formula V.

It is interesting that the carbinol-amine relationship discussed by Briggs, Newbold, and Stace (16) for solasodine (with the pyrrolizidine formula of Clemo *et al.* (13)) would be just as possible on the basis of the octahydro-

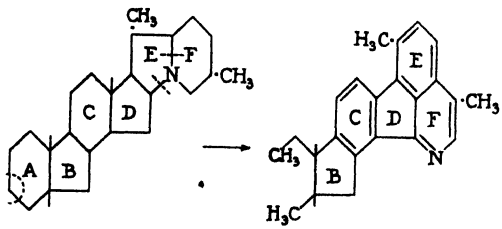
pyrrocoline formula, in which the suggested quaternary base, or carbinolamine, could have its OH group on either C atom 16 or 22. This would be analogous to the behavior of pseudostrychnine. Strychnine is now accepted as possessing a perhydropyrrocoline structure in its make-up. At this point, it may also be mentioned that there is a possibility that there can be a hitherto unsuspected analogy in the experience with the so called secondary base jervine to pseudostrychnine. This question is now under consideration.

Of further interest in the general picture is the nature of the more complex, basic dehydrogenation products, such as cevanthridine, which was one of the first crystalline products isolated by Blount (17) from the dehydrogenation of cevine. This substance cannot be considered entirely of primary character, but the result of partial cyclization during the dehydrogenation. In a previous publication, we presented data (18) which indicated that a fluorene ring system is also present in cevanthridine, which is in accord with the fluorene character of the hydrocarbons derived from the same dehydrogenation. The data also suggested that the N of cevanthridine may be contained in an isoquinoline ring. A possible mechanism for the formation of such a structure might be postulated as follows: The two bonds joining the nitrogen are first ruptured, as indicated in Formula VI. This is followed by synthesis of Ring E, as shown in Formula VII. There is, of course, precedent for this type of synthesis in the formation of the pentacyclic hydrocarbons formed during the dehydrogenation of various sterols. Once Ring E is formed, the synthesis of Ring F would not be unexpected, particularly if a hydroxyl were present on the carbon atom of Ring D involved in the ring closure. One of the remaining rings, perhaps Ring A, might possibly have been ruptured with loss of a C atom, and this would lead to the correct empirical formula. Another crystalline base, $C_{26}H_{25}N$ (?), isolated in the cevanthridine fraction (19), could possibly be one in which Ring A has not been opened.

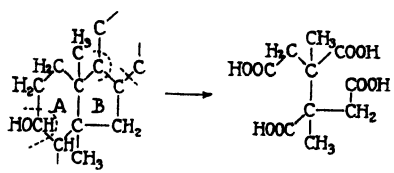
In the case of the dehydrogenation of solanidine, a good sized, tarry basic fraction also resulted. The various fractions obtained from it on refractionation at low pressure and a small amount which crystallized gave analytical figures of the same general order. In the case of the crystalline material, the analysis suggested solanthrene. But the melting point proved to be higher (183–197°). It is possible that aromatization of only one of the rings has occurred with loss of the angular methyl group on carbon atom 10, or that in some way a rearrangement has taken place at the N end of the molecule. No evidence of the production of any substance corresponding to cevanthridine could be obtained.

There remain to be discussed possible positions for the hydroxyl groups which occur in the various alkaloids. The fact that solanidine gives a

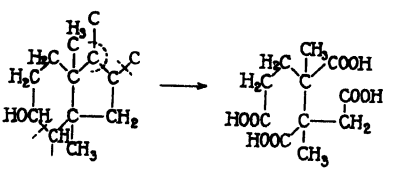
digitonide has shown that its hydroxyl group is undoubtedly in the 3 position. For the same reason, this must be the case in isorubijervine and rubijervine. It is almost certain that this position is also occupied by a hydroxyl group in the other veratrine alkaloids. Rubijervine appears to have its second hydroxyl group located in the vicinity of the first, since it gives the incompressible type of surface film (15). In jervine, in addition to the probable hydroxyl on position 3, a second one may be located on carbon atom 23, in order to permit the formation of the phenolic pyridine,



VI VII



VIII



IX

$C_8H_{11}ON$, isolated from the dehydrogenation products (7). There is no certain indication of the position of the third hydroxyl group. Aside from the probable one on carbon atom 3, cevine may have hydroxyl groups on carbon atom 21, as well as on carbon atom 23, in order to permit formation of the dicyclic pyridine, C_8H_9ON, previously reported. A fourth hydroxyl may be located on carbon atom 15 in order to account more easily for the formation of the assumed Ring F of cevanthridine, as outlined above. If the structures and origin of the hexanetetracarboxylic and

heptanetetracarboxylic acids obtained from cevine (11) have been correctly interpreted as products of cleavage of Ring A and a 5-membered Ring B, as shown in Formulas VIII and IX, then carbon atoms 1, 2, and 7 could not be hydroxylated.

The exact position of the double bond in the various alkamines must be a subject for subsequent discussion. The usual 5,6 position, which has been assumed in the case of solanidine, could not be considered with the veratrine alkamines, if the assumption of the angular methyl group on carbon atom 5 is correct. It appears possible that the double bond in some of the veratrine bases may occupy a different position than in the corresponding iso bases. With the exception of jervine, none of these bases gave a positive Rosenheim reaction with trichloroacetic acid. Finally, it has now been found that isogermine can be produced directly from germine by isomerization with alkali, as already reported in the conversion of protoverine to isoprotoverine (6). However, similar attempts to isomerize rubijervine or isorubijervine have been thus far unsuccessful.

TABLE II

Veratrine Bases and Digitonin

Base	Weight	Digitonide obtained	Digitonide, theory
	gm.	*gm.*	*gm.*
Isorubijervine	0.0113	0.0341	0.0450
Rubijervine	0.0115	0.0232	0.0455
Jervine	0.0119	None	
Cevine	0.0147	"	
Protoverine	0.0146	"	
Isogermine	0.0143	"	
Germine	0.0143	"	

EXPERIMENTAL

Veratrine Bases and Digitonin—In Table II are recorded the results obtained by mixing solutions of the alkaloid bases and digitonin. In each case, the alkamine was dissolved in 1.5 cc. of 95 per cent alcohol, and to the warm solution 4 cc. of a 1 per cent solution of digitonin (S. B. Penick and Company) in 90 per cent alcohol were added. In the case of isorubijervine, crystallization of the digitonide as needles was copious within the 1st hour. The rubijervine mixture remained clear until the next day when micro needles had begun to deposit. The results reported were obtained after 48 hours standing.

Dihydrogermine—A solution of 1 gm. of germine in 80 cc. of butanol was heated to boiling. 4 gm. of sodium were added and the mixture was

vigorously shaken. After completion of the reaction, water was added and CO_2 was passed through in excess. Following concentration *in vacuo* to remove the butanol, water was added and sufficient dilute NaOH to dissolve any precipitated $NaHCO_3$. The solution was extracted with chloroform in a continuous extractor for 20 hours. On concentration of the chloroform solution to about 10 cc., a copious crystalline mixture was obtained. The collected material amounted to 0.44 gm. On recrystallization from methanol, it formed mostly four-sided micro leaflets, which gradually darkened and shrank above 258° to a resin which finally melted at 265° (uncorrected).[1] The following rotation was calculated for the dried substance.

$$[\alpha]_D^{28} = -57° \ (c = 0.93 \text{ in pyridine})$$

For analysis, it was dried at 110° and 2 mm.

$C_{27}H_{45}O_8N$.	Calculated.	C 63.36, H 8.87
	Found. (*a*)	" 62.95, " 8.73
	" (*b*)	" 63.33, " 8.81
	" (*c*)	" 63.34, " 8.97

The Tschugaeff-Zerewitinoff determination was as follows on dried substance: 3.493 mg. of substance gave 1.44 cc. of CH_4 (26.5°, 742 mm.); there was no change at 95°. Found, H 1.65; calculated for 8H, 1.58.

The *hydrochloride* was obtained from methanol-ether as minute, short micro prisms, which melted with decomposition above 250° (uncorrected) after preliminary discoloration.

For analysis, the substance was dried at 110° and 2 mm.

$C_{27}H_{46}O_8NCl$.	Calculated.	C 59.14, H 8.46, Cl 6.47
	Found.	" 59.27, " 8.28, " 6.37

Isomerization of Germine. Isogermine—0.2 gm. of germine was dissolved by warming in a mixture of 1 cc. of methanol, 3 cc. of H_2O, and 1 cc. of N NaOH. The mixture was cooled and maintained at 50° for 6 hours. The solution, which had become yellow, was extracted in a continuous extractor with chloroform for 20 hours. The extract was concentrated to a few cc. and seeded. A crop of needles, which consisted essentially of the chloroform compound of germine, was obtained (0.15 gm.).

The mother liquor was concentrated and, after addition of methanol, the remaining chloroform was boiled off. The concentrated solution crystallized readily when seeded with isogermine as needles. The separation was aided by careful addition of ether. The yield was 30 mg. After recrystallization, it melted gradually at 258–260° (uncorrected) after pre-

[1] Uncorrected melting points were taken in the usual manner, but not corrected for stem exposure. The others are corrected micro melting points.

liminary sintering and discoloration, especially above 250°. It agreed in all properties with the isogermine previously described (1).

$[\alpha]_D^{27} = -46.6°$ ($c = 1.03$ in 95% alcohol)
$[\alpha]_D^{28} = -56.0°$ ($c = 1.00$ in pyridine)
$C_{27}H_{43}O_8N$. Calculated, C 63.61, H 8.51; found, C 63.44, H 8.50

Dihydroisogermine—0.2 gm. of isogermine, when shaken in methanol with 50 mg. of platinum oxide catalyst, gradually dissolved as hydrogenation proceeded. Approximately 1 mole of hydrogen in excess of the catalyst requirements was absorbed. The filtrate from the catalyst was diluted and, when the attempt was made to extract the product with chloroform, a sparingly soluble chloroform compound separated as small flat needles. The collected substance was dissolved in hot 95 per cent alcohol and concentrated to smaller volume. After addition of ether, although at first clear, a gelatinous mass began to form which on warming gradually transformed to a paste of delicate or broader flat needles. After collection with an alcohol-ether mixture, the substance darkened, especially above 265°, and melted after preliminary softening at 277–278° (uncorrected). The following rotation was calculated for the dried substance.

$[\alpha]_D^{28} = -61°$ ($c = 0.95$ in pyridine)

For analysis, it was dried at 120° and 0.2 mm.

$C_{27}H_{45}O_8N$.	Calculated.	C 63.36, H 8.87
	Found. (*a*)	" 62.90, " 9.05
	" (*b*)	" 63.51, " 8.94

The Tschugaeff-Zerewitinoff determination was as follows on dried substance: 3.985 mg. of substance gave 1.55 cc. of CH_4 (26.5°,742 mm.); there was no change at 95°. Found, H 1.56; calculated, 8H 1.58.

The concentrated solution of this substance in methanol did not behave like dihydrogermine, and could not be seeded with the latter.

Dihydrorubijervine—0.1 gm. of rubijervine was dissolved in a small volume of methanol with the aid of a slight excess of acetic acid, and shaken with 50 mg. of platinum oxide catalyst and H_2. The absorption was roughly equivalent to 1 mole of H_2 in excess of the catalyst requirements. After filtration, partial concentration, and dilution, the addition of Na_2CO_3 solution caused separation of a gelatinous mass which was extracted with chloroform The extract yielded a residue which crystallized from 95 per cent alcohol as delicate needles, which melted at 222° (uncorrected).

It contained solvent and was dried for analysis at 120° and 0.2 mm.

$C_{27}H_{45}O_2N$. Calculated, C 78.01, H 10.92; found, C 78.45, H 11.09

Diacetyldihydrorubijervine—Dihydrorubijervine was refluxed with acetic anhydride for 1 hour and evaporated to dryness. The crystalline residue

dissolved completely in water which contained a slight excess of H_2SO_4. After precipitation with dilute Na_2CO_3, the base was extracted with chloroform. The extracted substance crystallized readily from 95 per cent alcohol as needles which melted at 216–219° (uncorrected).

$C_{31}H_{49}O_4N$. Calculated, C 74.49, H 9.89; found, C 74.85, H 9.87

Dihydroisorubijervine—0.1 gm. of isorubijervine was hydrogenated as in the case of rubijervine. The absorption of H_2 was approximately 1 mole for the substance. The base was extracted with chloroform and crystallized from methanol as compact, often triangular or trapezoidal prisms, which melted at 244° (uncorrected) after slight preliminary softening.

$C_{27}H_{45}O_2N$. Calculated, C 78.01, H 10.92; found, C 77.85, H 10.99

Dehydrogenation of Solanidine—A mixture of 10.1 gm. of solanidine and 30 gm. of selenium was heated in a flask, after the air was replaced by nitrogen, at 340° for 2 hours. The oil which distilled into a chilled receiver through an outlet tube a few cm. above the bath was set aside. The investigation of this material has been reported previously (3). The residue was combined with similar material from a preliminary run of 2 gm., powdered, and exhaustively extracted with ether. Upon evaporation of the extract, a residue of 8.6 gm. was obtained. This was redissolved in ether and shaken out with 20 cc. of 10 per cent HCl. The aqueous layer which contained tar was set aside to be treated as described below. The ether solution was washed again with dilute HCl, dried over K_2CO_3, and evaporated to dryness. 3.5 gm. of oil remained.

This residue in benzene solution was chromatographed through 80 gm. of alumina (Merck). The first 75 cc. of eluent (benzene), after material began to emerge from the chromatograph, contained 2.35 gm. of substance which was scarcely retained by the adsorbent. This proved to be the hydrocarbon fraction which was fractionated as described below. About 0.3 gm. of additional material was eluted by benzene in several succeeding fractions. None of these could be obtained crystalline. They were not investigated further. Methanol subsequently eluted about 0.4 gm. of colored material, the major portion of which was sublimed at low pressures. Analysis of the sublimate gave figures for oxygen-containing material (found, C 83.19, H 8.35) which could not be crystallized.

The above hydrocarbon fraction was placed in a sublimation apparatus and all was collected which distilled up to an oil bath temperature of 200° under a pressure of 0.3 mm. The entire distillate was then fractionated in a micro fractionation apparatus with a column 21 cm. in length. The pressure throughout was 0.25 mm. The fractionation is recorded in Table III.

2-Methylphenanthrene—Both Fractions 4 and 5 contained this hydrocarbon, but it could not be isolated from either Fraction 3 or 6. The crude fraction was treated with 60 mg. of picric acid and crystallized at 0° from a mixture of about 3 parts of ether to 1 of acetone. Characteristic yellow, delicate, curved needles separated, which after two recrystallizations melted at 120–121°.

$C_{15}H_{12} \cdot C_6H_3O_7N_3$. Calculated, C 59.85, H 3.59; found, C 59.73, H 3.42

TABLE III

Fractionation of Hydrocarbons from Solanidine

Fraction No.	Bath temperature	Column temperature	Weight (approximate)	Character and m.p	Analysis	
					C	H
	°C.	°C.	mg.		per cent	per cent
1	160	110	80	Oil		
2	170	120	70	"		
3	175	130	70	"		
4	175	126	100	"	90.50	9.27
5	173	130	100	"		
6	180	135	100	"	90.44	9.19
7	184	140	150	Partly crystalline		
8	190	140	150	Crystalline; 70–140°	91.89	8.13
9	195	145	130	"	91.78	8.28
10	200	170	130	Partly crystalline		
11	210	170	130	Crystalline	92.85	7.07
12	230	170	130	" 80–100°		
13	230	172	150	" 105–120°	92.93	7.02
14	230	180	130	Partly crystalline		
15	250	220	100	Oil		

The hydrocarbon was regenerated from the picrate and, after recrystallization from isopentane, formed tightly packed, flat blades which melted at 57–59°.

$C_{15}H_{12}$. Calculated, C 93.71, H 6.29; found, C 93.46, H 6.42

1,2-Dimethylphenanthrene—Fractions 7, 8, and 9, upon recrystallization from ether, gave in each case the same hydrocarbon which crystallized in lustrous, thin leaves. It melted at 146–148°, and showed no depression when mixed with a sample of synthetic 1,2-dimethylphenanthrene. The crystalline form and properties appeared to be identical.

$C_{16}H_{14}$. Calculated, C 93.15, H 6.85; found, C 93.14, H 6.74

The picrate from acetone formed rather stout, light orange needles which melted at 157–158°.

$C_{16}H_{14} \cdot C_6H_3O_7N_3$. Calculated, C 60.66, H 3.93; found, C 60.90, H 4.08

γ-Methyl-1,2-cyclopentenophenanthrene—This hydrocarbon comprised the major portion of Fractions 11 to 14 and was readily recrystallized from ether. It formed characteristic thin leaves which melted at 126–127°, and showed no depression when mixed with Diels' hydrocarbon, which had been obtained by the dehydrogenation of strophanthidin. It also showed an identical blue fluorescence in ultraviolet light.

$C_{18}H_{16}$. Calculated, C 93.05, H 6.95; found, C 93.15, H 6.95

The symmetrical trinitrobenzene derivative after recrystallization from acetone formed characteristic yellow needles, which melted at 151–152°.

$C_{18}H_{16} \cdot C_6H_3O_6N_3$. Calculated, C 64.72, H 4.30; found, C 64.72, H 4.08

TABLE IV

Fractionation of High Boiling Bases

Fraction No.	Bath temperature	Column temperature	Weight (approximate)	Character	Analysis	
					C	H
	°C.	°C.	*mg.*		*per cent*	*per cent*
1	243	210	150	Oil mixed with Se		
2	243	210	150	Clear resin		
3	243	214	150	" "	85.72	10.57
4	243	214	150	" "		
5	243	214	150	" "		
6	247	219	150	" "		
7	247	219	150	" "		
8	247	219	150	" "	85.85	10.35
9	247	219	150	" "		
10	247	219	150	" "		
11	247	219	150	" "		
12	247	219	150	Few crystals		
13	258	219	100	" "	85.72	10.13

The above acid aqueous layer from the original ether extract of the selenium residues was extracted with chloroform. This removed all the suspended tar. No appreciable material remained in the aqueous phase. The chloroform extract was shaken with NaOH solution to reconvert to the free base. Inappreciable amounts of phenolic material were removed by the alkali. The washed chloroform extract on concentration yielded 5 gm. of a dark residue. This was dissolved in 40 cc. of benzene and chromatographed through 100 gm. of alumina. The material, which was little adsorbed and passed quickly through with subsequent elutions with benzene, weighed 3.25 gm. This will be described below. Following this, a much smaller fraction was eluted very slowly by benzene, but this material could not be induced to crystallize. 1.4 gm. of unstable, oxygen-

containing, and dark colored material were then removed with methanol. It was not suitable for further investigation.

The above 3.25 gm. were distilled in the sublimation apparatus under 0.2 mm. pressure. 2.9 gm. were collected up to an oil bath temperature of 220°. This material was fractionated in a micro fractionation apparatus with a 21 cm. column, as recorded in Table IV. The pressure throughout the fractionation was 0.25 mm.

Fraction 13 was recrystallized from acetone-ether. 50 mg. of leaflets were obtained, which melted at 145–190°. After recrystallization from chloroform at −15°, 15 mg. of diamond-shaped leaflets were collected, which melted at 183–197°. Lack of material prevented further recrystallization.

$C_{27}H_{41}N$.	Calculated.	C 85.41, H 10.89
$C_{26}H_{37}N$.	"	" 85.88, " 10.26
	Found.	" 85.35, " 10.71

All analyses and active H determinations were kindly performed by Mr. D. Rigakos.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **148**, 41 (1943). Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **148**, 57 (1943).
2. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **148**, 51 (1943).
3. Craig, L. C., and Jacobs, W. A., *Science*, **97**, 122 (1943). Prelog, V., and Szpilfogel, S., *Helv. chim. acta*, **25**, 1306 (1942).
4. Bergel, F., and Wagner, R., *Ber. chem. Ges.*, **66**, 1093 (1933).
5. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **125**, 625 (1938).
6. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **149**, 271 (1943).
7. Jacobs, W. A., Craig, L. C., and Lavin, G. I., *J. Biol. Chem.*, **141**, 51 (1941). Saito, K., Suginome, H., and Takaoka, M., *Bull. Chem. Soc. Japan*, **11**, 172 (1936).
8. Soltyz, A., and Wallenfels, K., *Ber. chem. Ges.*, **69**, 811 (1936).
9. Rochelmeyer, H., *Arch. Pharm.*, **274**, 543 (1936).
10. Ruzicka, L., Ehmann, L., Goldberg, M. W., and Hösli, H., *Helv. chim. acta*, **16**, 833 (1933).
11. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **141**, 253 (1941).
12. Marker, R. E., and Rohrmann, E., *J. Am. Chem. Soc.*, **61**, 846 (1939).
13. Clemo, G. R., Morgan, W. M., and Raper, R., *J. Chem. Soc.*, 1299 (1936).
14. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **120**, 447 (1937).
15. Rothen, A., and Craig, L. C., *J. Am. Chem. Soc.*, in press.
16. Briggs, L. H., Newbold, R. P., and Stace, N. E., *J. Chem. Soc.*, 3 (1942).
17. Blount, B. K., *J. Chem. Soc.*, 124 (1935).
18. Craig, L. C., Jacobs, W. A., and Lavin, G. I., *J. Biol. Chem.*, **139**, 277 (1941). Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **139**, 293 (1941).
19. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **139**, 263 (1941).

THE STABILITY OF CAROTENE IN ACETONE AND PETROLEUM ETHER EXTRACTS OF GREEN VEGETABLES*

I. THE PHOTOCHEMICAL DESTRUCTION OF CAROTENE IN THE PRESENCE OF CHLOROPHYLL

II. THE STABILIZING EFFECT OF SODIUM CYANIDE

By LEONARD P. PEPKOWITZ

(From the Department of Agricultural Chemistry, Rhode Island Agricultural Experiment Station, Kingston)

(Received for publication, May 12, 1943)

I. The Photochemical Destruction of Carotene in the Presence of Chlorophyll

The photochemical destruction of carotene in the presence of chlorophyll was first observed when acetone extracts of green leafy vegetables were allowed to stand for a 4 hour period on a laboratory bench exposed to sunlight. Subsequently it was found that the destruction of the carotene was much more rapid in the extracts of cooked samples than in extracts of raw samples. A loss of as much as 60 per cent of the carotene was noted in the extracts of cooked material and a 40 per cent loss of carotene in the extracts from raw material, during a 6 hour exposure to ordinary daylight. A practical application of the observations described in this paper is the necessity for the protection of plant extracts from undue exposure to light or the immediate removal of chlorophyll from such extracts during the course of a series of carotene estimations, since in most of the current methods of carotene analysis petroleum ether or acetone is used as the extracting medium.

Accordingly the following experiments were devised to substantiate these observations. Acetone extracts of a number of different green vegetables were prepared by the use of the Waring blendor. 30 gm. samples of the fresh vegetables were used. One sample was cooked before extraction; to a second, 50 mg. of sodium cyanide were added before extraction; a third sample remained untreated, and will hereafter be referred to as the "raw sample." Aliquots of the various extracts were taken; half were exposed to sunlight, and the other half were placed in the dark. The carotene estimations were made by the method of Petering, Wolman, and Hibbard (1).

The carotene contents after stated periods of time are given in Table I. It will be observed that in all cases, except for raw carrots, there were

* Contribution No. 646 of the Rhode Island Agricultural Experiment Station.

decided losses of carotene upon exposure to light, which did not take place in the dark, and also that the losses of carotene in the extracts of the cooked samples were much greater than in the extracts of the raw vegetables. The effect of cyanide will be discussed in Part II.

TABLE I

Photochemical Destruction of Carotene in Raw and Cooked Vegetables during 6 Hour Exposure to Light

Vegetable	Treatment	Carotene per 100 gm. fresh weight			Loss in light
		Original	In dark	In light	
		mg.	*mg.*	*mg.*	*per cent*
Spinach	Raw	3.6		2.2	39
	" + NaCN	3.9		3.1	21
	Cooked	3.8		1.5	61
Endive	Raw	3.3		1.6	52
	" + NaCN	3.6		2.4	33
	Cooked	3.5		1.3	63
"	Raw	3.0	3.4	1.6	47
	" + NaCN	3.2	3.8	2.5	22
	Cooked	3.3	3.9	1.3	61
Broccoli	Raw	3.4	3.2	2.7	36
	" + NaCN	4.2	4.7	2.7	36
	Cooked	3.7	3.4	1.7	54
Pepper	Raw	0.40	0.45	0.25	38
	" + NaCN	0.45	0.48	0.23	49
	Cooked	0.48	0.50	0.13	71
Carrot*	Raw	6.2	6.3	6.4	0
	" + chlorophyll	6.2	6.3	3.8	40

* Exposure period, 4 hours.

TABLE II

Destruction of Pure Carotene in Petroleum Ether Solution of Chlorophyll Exposed to Light for 6 Hour Period

Original	Found	Loss
mg.	*mg*	*per cent*
0.25	0.11	56.0
0.63	0.25	60.3
1.26	0.54	57.1

The reasons for the greater loss of carotene in the extracts of the cooked samples are not apparent but it might be assumed that some inhibitor, that might prevent the destruction of carotene, is destroyed during the cooking process. However, this was not substantiated when cooked and

raw samples were exposed to light before extraction, for no loss of carotene was observed even after extended exposure. The loss of carotene apparently took place only after extraction, and not in the tissue itself.

Carrots were included in Table I because they offer a good example of the phenomenon being discussed. The roots of carrots possess no chlorophyll; therefore extracts of this vegetable do not lose carotene upon exposure to light. However, if chlorophyll is added to the carrot root extract, carotene is destroyed, losing 40 per cent of the original carotene content after a 4 hour exposure to light. The importance of light is indicated by the fact that carrot extract plus chlorophyll in the dark lost no carotene during the 4 hour interval.

The destruction of carotene was not confined to acetone solutions alone. Pure carotene dissolved in petroluem ether (b.p. 30–60°) solution of carotene-free chlorophyll showed a similar loss of carotene upon exposure to light, as is indicated in Table II.

By means of the following experiment the interdependent effects of chlorophyll and light in the photochemical destruction of carotene were demonstrated. A carotene-free solution of chlorophyll was prepared by extracting the tops of potato plants with acetone in a Waring blendor and filtering the extract. The filtrate was shaken with one-fourth its volume of petroleum ether in a large separatory funnel, dissolving the carotene in the petroleum ether layer while the chlorophylls and carotenoid pigments were partitioned in the two layers. The petroleum ether layer was evaporated to dryness, the residue taken up with acetone, and a second extraction with petroleum ether was made. The two acetone layers were combined and evaporated to the desired concentration of chlorophyll. This procedure did not give a quantitative separation of the chlorophyll, as the greater part of the original chlorophyll content remained in the petroleum ether layer. However, sufficient chlorophyll, free from carotene but containing xanthophylls and flavones, was obtained in an acetone solution. Since the chlorophyll solution could not be evaluated as to its actual chlorophyll content, no appropriate standard being available, the various concentrations of chlorophyll utilized in the experiments will be referred to in terms of ml. of the chlorophyll solution taken.

Known aliquots of pure carotene (S. M. A. Corporation) in acetone were placed in test-tubes, 5 ml. of the carotene-free chlorophyll solution were added to one-half of the test-tubes, and all brought to the same volume with acetone. The test-tubes were equally divided; half were placed in the dark and the rest exposed to sunlight. Thus four different treatments were provided: carotene plus chlorophyll in the light; carotene minus chlorophyll in the light; carotene plus chlorophyll in the dark; carotene minus chlorophyll in the dark. Each treatment was conducted in dupli-

cate for two different time periods. The results of three separate experiments are given in Table III.

It will be observed that the presence of both light and chlorophyll is necessary for the rapid destruction of carotene to take place. It must be emphasized that the phenomenon described in this paper is separate from the known enzymatic oxidation of carotene (2–4). In the absence of either chlorophyll or light, or both, no loss of carotene could be observed under the experimental conditions employed. Furthermore, as indicated in Table III, the reaction was a function of time, since the longer the exposure of the extracts to light, the greater was the loss of carotene.

The quantity of chlorophyll present was also a factor in the photochemical destruction of carotene, for the results in Table IV indicate that increasing the quantity of chlorophyll further increased the loss of carotene over a given period of time. The direct dependency of the destruction

TABLE III

Photochemical Destruction of Carotene As Function of Chlorophyll and Light

Carotene taken	In light		In dark	
	Plus chlorophyll	Minus chlorophyll	Plus chlorophyll	Minus chlorophyll
mg	*mg.*	*mg.*	*mg*	*mg.*
0.71 (2 hrs.)	0.29	0.70	0.70	0.72
0.73 (4 ")	0.13	0.73	0.71	0.73
0.38 (6 ")	0.15	0.37	0.36	0.38
0.38 (8 ")	0.08		0.38	0.38
0.66 (2 ")	0.42		0.67	
0.66 (4 ")	0.23		0.63	

of carotene upon the quantity of chlorophyll present when exposed to light would appear to indicate that the chlorophyll was directly involved in the reaction, and does not act merely as a catalyst. The mechanism of the photochemical reaction is unknown, for the products of the reaction have not been determined. The data of Table IV reemphasize the fact that the reaction is a function of time, since there was a greater loss at the end of 6 hours than at the end of 3 hours.

That chlorophyll alone was effective in the photochemical destruction of carotene and not the accompanying xanthophyll and flavone pigments was demonstrated in a subsequent experiment by removal of the chlorophyll by adsorption on barium hydroxide (1) in half of the samples, leaving only the carotenoid pigments in solution. With 0.17 mg. of pure carotene, the solution containing chlorophyll dropped to 0.01 mg. of carotene, while the solution containing the carotenoids had 0.16 mg. of carotene after a

2 hour exposure to light. In a repetition of this experiment with 0.31 mg. of carotene, at the end of 2 hours the chlorophyll solution dropped to 0.14 mg., while the solutions with the carotenoid pigments still possessed 0.28 mg. of carotene. It is obvious from these data, as well as the data for carrot roots in Table I, that chlorophyll alone was the activating agent in the observed photochemical destruction of carotene.

Chlorophyll has been found to be an activating agent for the photochemical oxidation of other compounds. Meyer (5) has shown this to be true for the photochemical oxidation of such compounds as oleic acid, lycopene, citronellal, linalool, pulegone, and ergosterol. Gaffron (6) presents evidence which indicates that autoxidations involving chlorophyll proceed through the formation of secondarily produced peroxides and are therefore coupled reactions. The non-photochemical enzymatic oxidation of carotene in the presence of unsaturated fats has been shown by

TABLE IV

Effect of Different Concentrations of Chlorophyll on Photochemical Destruction of Carotene

Chlorophyll added	3 hrs		6 hrs	
	Carotene	Loss	Carotene	Loss
ml.	*mg.*	*per cent*	*mg.*	*per cent*
0	0.23	0.0	0.29	0.0
5	0.12	47.8	0.07	75.8
10	0.06	73.9	0.04	86.2
20	0.03	87.0	0.02	93.1
30	0.04	82.6	0.01	96.6

Sumner (2) to be caused also by a coupled reaction through the formation of a reactive intermediate primary oxide. It is of interest in this connection that Aronoff and Mackinney (7) have shown that carotene has an inhibitory effect on the photochemical destruction of chlorophyll even below 5500 Å., where carotene does not absorb light.

II. The Stabilizing Effect of Sodium Cyanide

Two effects of the addition of sodium cyanide are indicated by the data of Table I. First, sodium cyanide has a partial inhibitory action upon the photochemical destruction of carotene in the presence of chlorophyll, since in almost all cases the quantity of carotene destroyed is less in the presence of cyanide than in its absence. Because the mechanism involved in the reaction described is unknown, it is difficult to evaluate the function of the added cyanide in prevention of the destruction of carotene. However, the ability of cyanide to act as a catalytic poison is well established

and the assumption may be hazarded that at least one step in the complete reaction may well be enzymatic, but whether the enzymatic action is a coupled reaction as discussed above is not indicated by the data.

The second effect of the sodium cyanide also involves its inhibitory action on enzymes. The reaction in question, although incurring a loss of carotene, is separate from the photochemical destruction of carotene referred to in Part I of this paper. Previous experience, as well as reports in the literature (8–10), has shown that there is a discrepancy between the estimation of carotene in raw and cooked vegetables. The carotene content calculated from raw samples was almost invariably lower than that from cooked samples. This difference appears to be caused by an enzymatic destruction of carotene during the maceration of the raw vegetable, and

TABLE V

Prevention of Loss of Carotene by Addition of Sodium Cyanide to Acetone Extracts of Raw Vegetables

Vegetable	Carotene per 100 gm fresh weight		
	Raw	Cooked	Raw + NaCN
	mg.	*mg*	*mg.*
Pepper*	0.44	0.55	
" *	0.28	0.67	
Spinach*	2.1	3.3	
"	3.5	3.8	3.9
Endive	3.4	3.9	3.8
Broccoli	3.2	4.5	4.7
"	3.6	4.5	4.2
Pepper	0.40	0.50	0.48
"	0.38	0.55	0.58

* Values determined before the effect of added NaCN was observed.

cooking evidently inactivates this enzyme. Strain (11) previously observed this phenomenon. This author found that the oxidation of the carotenoid pigments in macerated etiolated and green tissue was inhibited by heating the tissue before grinding, or by the addition of small quantities of zinc dust, zinc oxide, magnesium oxide, sodium hydroxide, ammonium hydroxide, copper sulfate, lead acetate, and mercuric chloride. However, conditions under which enzymes were not destroyed, such as freezing, did not retard the oxidation of the carotenoid pigments. In the present case, the addition of 50 mg. of sodium cyanide for 30 to 50 gm. of the vegetable, just previous to extraction, eliminated this difference in the carotene estimation and brought the results obtained with raw and cooked vegetables into agreement, as indicated by the data of Table V.

A similar response, although not as uniformly effective, was obtained by using iodoacetic acid instead of sodium cyanide, but the use of antioxidants such as diphenylamine or sodium acid sulfite proved valueless.

SUMMARY

1. Carotene is destroyed when dissolved in acetone or petroleum ether and exposed to light in the presence of chlorophyll. This reaction has been demonstrated in extracts of green vegetables. The importance of this fact in the estimation of carotene is indicated.

2. The reaction is a function of time; the longer the exposure to light, the greater the loss of carotene.

3. Both light and chlorophyll are necessary for the reaction to occur. In the absence of chlorophyll, or light, or both, no loss of carotene could be observed.

4. The rate of destruction of carotene is dependent upon the quantity of chlorophyll present. Thus chlorophyll is directly involved in the reaction and does not act merely as a catalyst.

5. The addition of sodium cyanide partially inhibits the photochemical destruction of carotene.

6. Sodium cyanide also prevents the non-photochemical enzymatic destruction of carotene, which has been observed to occur in extracts of raw vegetables.

The author wishes to express his indebtedness to Professor John B. Smith for his many helpful suggestions during the course of these experiments and in preparation of the paper.

BIBLIOGRAPHY

1. Petering, H. G., Wolman, W., and Hibbard, R. P., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 148 (1940).
2. Sumner, R. J., *J. Biol. Chem.*, **146**, 215 (1942).
3. Tauber, H., *J. Am. Chem. Soc.*, **62**, 2251 (1940).
4. Quackenbush, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, **145**, 169 (1942).
5. Meyer, K., *J. Biol. Chem.*, **103**, 597, 607 (1933).
6. Gaffron, H., *Biochem. Z.*, **264**, 251 (1933).
7. Aronoff, S., and Mackinney, G., *J. Am. Chem. Soc.*, **65**, 956 (1943).
8. Zimmerman, W. I., Tressler, D. K., and Maynard, L. A., *Food Research*, **5**, 93 (1940).
9. Fraps, G. S., Meinke, W. W., and Kemmerer, A. R., *J. Assn. Off. Agric. Chem.*, **24**, 739 (1941).
10. Booher, L. E., and Marsh, R. L., *U. S. Dept. Agric., Tech. Bull. 802* (1942).
11. Strain, H., Leaf xanthophylls, *Carnegie Inst. Washington, Pub. No. 490* (1938).

THE BIOLOGICAL ACTIVITY OF VITAMIN A_2*

By JAMES L. JENSEN, EDGAR M. SHANTZ, NORRIS D. EMBREE, JOHN D. CAWLEY, AND PHILIP L. HARRIS

(From the Laboratories of Distillation Products, Inc., Rochester, New York)

(Received for publication, June 11, 1943)

In 1937 Lederer *et al.* (1) reported that the livers of certain fresh water fish contained a substance giving an absorption maximum at 693 mμ when treated with antimony trichloride. This observation was confirmed by Edisbury *et al.* (2), who designated this material vitamin A_2. Further work by Gillam *et al.* (3) indicated that vitamin A_2 possessed biological activity for rats and Lederer and Rathman (4) have shown that vitamin A_2 is absorbed and stored in the liver by rats and frogs. On the other hand, Karrer *et al.* (5) have recently stated that vitamin A_2 does not have biological activity for rats.

We feel that this disagreement is due primarily to the different methods employed by the above workers to calculate the relative amounts of vitamins A_1 and A_2 in their preparations. In each case, this calculation has been made from the ratio of E (695 mμ) to E (620 mμ) of the preparation, but there is a divergence of opinion concerning the value for this ratio for each of the pure vitamins. The crystalline preparations of Baxter and Robeson (6) have allowed an accurate measurement of this value for vitamin A_1, but a similarly exact calculation for vitamin A_2 has, until the present, not been possible. The values that have been assumed are probably incorrect, as will be shown below. Since neither Gillam nor Karrer gave the physicochemical properties of the concentrates actually assayed, we have bioassayed a vitamin A_2 concentrate whose physicochemical characteristics were measured by us.

EXPERIMENTAL

Preparation of Vitamin A_2 Solution—The vitamin A_2 which was assayed for vitamin A activity was prepared as follows: The livers from seven freshly caught northern pike (*Esox lucius*) were finely minced in a high speed mixer, diluted with water, and extracted five times with ethyl ether. 20 gm. of oil were obtained from 400 gm. of liver. The absorption maximum in the ultraviolet region was at 352 mμ with E (1 per cent, 1 cm.) = 48.9. A subsidiary maximum occurred at 286 mμ with E (1 per cent, 1 cm.) = 20.6. With a saturated solution of antimony trichloride in chloroform, the oil gave a greenish blue color having an absorption maxi-

* Communication No. 36 from the Laboratories of Distillation Products, Inc.

mum at 695 mμ with E (1 per cent, 1 cm.) = 166. The ratio E (695 mμ) to E (620 mμ) of this oil was 3.01.

The liver oil was then subjected to distillation in a cyclic molecular still after first being dissolved in a carrier oil.[1] The fraction distilling from 180–220° was taken for biological assay. This fraction, weighing 12.9 gm., contained about 50 per cent of the vitamin A_2 present in the original liver oil. It had an ultraviolet absorption maximum at 352 mμ with E (1 per cent, 1 cm.) = 39.0 and a subsidiary maximum at 286 mμ with E

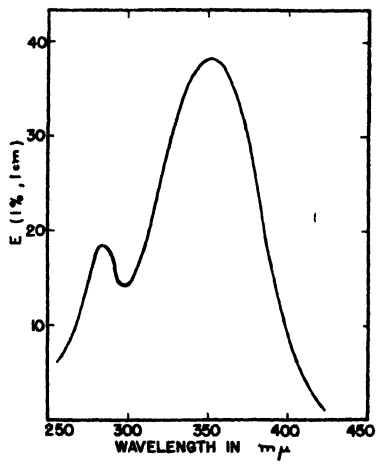


FIG. 1

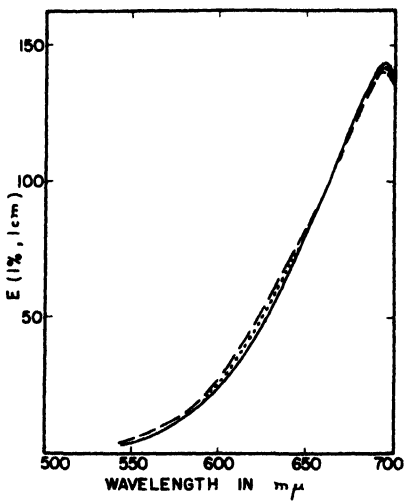


FIG. 2

FIG. 1. Absorption spectrum of distillate from liver oil of northern pike (in ethanol).

FIG. 2. Absorption spectrum of the antimony trichloride product of pike liver oil distillate (solid line). The absorption spectrum (× 525) of the antimony trichloride product of the liver extract from rats fed vitamin A_2 (dash line) and the absorption spectrum (× 0.04) of the antimony trichloride product of chromatographically pure anhydro vitamin A_2 (dotted line) are shown for comparison.

(1 per cent, 1 cm.) = 18.6 (Fig. 1). The antimony trichloride product had an absorption maximum at 695 mμ with E (1 per cent, 1 cm.) = 143 (Fig. 2). The ratio E (695 mμ) to E (620 mμ) of this fraction was 3.06.

Biological Assay—The biological assay was carried out by the procedure of the United States Pharmacopoeia XII (7). Three levels of reference

[1] A triglyceride oil composed of corn oil residue and a mixture of synthetic glycerides distilling over a wide temperature range. The purpose of this oil is to increase the bulk of both the distilland and the distillates so that they can be more easily handled in the molecular still.

oils were used, 1, 2, and 4 U.S.P. units per rat per day. These doses gave respectively 21.5, 37.6, and 65.5 gm. average gain in weight per rat for the 28 day period. The vitamin A_2 concentrate was fed at three levels, 25.64, 128.2, and 1282.0 mg. per rat per day. The average growth responses for these levels were 25.8, 54.7, and 60.7 gm., respectively. A dose-response curve from the reference oil data was determined. It is given by the formula $\log X = (Y - 19.5)/73.1$, where X equals the units fed per day and Y equals the growth response in gm. during the 28 day experimental period. This formula and the growth response for the 25.64 mg. level of vitamin A_2 concentrate indicated that it has a potency of 47,500 U.S.P. units per gm.

The livers of the rats fed the higher doses of the vitamin A_2 concentrate were examined for vitamins A_1 and A_2 after extraction in the same manner as the fish livers. The antimony trichloride reaction product (Fig. 2) showed the presence of only vitamin A_2; the ratio of E (695 mμ) to E (620 mμ) was 3.03.

Toxicity of Vitamin A_2—The growth responses of 54.7 and 60.7 gm., obtained by the higher doses of the vitamin A_2 concentrate, are difficult to interpret. Gillam *et al.* observed a similar phenomenon when they found that increasing doses of liver oil from *Lucioperca lucioperca*, 0.6 to 4.8 units per day, induced practically the same average gain in weight in their prophylactic type of bioassay. This depressed growth response at relatively high levels of vitamin A_2 feeding is puzzling, and to quote their paper:

"Whilst this might have been due in part to the fact that all the rats in this group (cf. Table IV) were exclusively female whilst all the others were males, it is significant that we have obtained similar results with *Silurus* concentrates when all the rats were of the same sex. We are inclined to attribute these results to some toxic property of the fresh water fish oils, but propose to examine the point further. It is of some interest to note that the livers of *Huso huso* and *Silurus glanis* are traditionally regarded as poisonous by the Volga fishermen, but this cannot be accepted as evidence without further tests."

Our bioassay data contribute no positive evidence for toxicity of vitamin A_2. However, we have compared the growth response of normal weanling rats fed daily for a 2 week period 10,000 units (1 gm. of oil solution with E (1 per cent, 1 cm.) (328 mμ) = 5) of vitamin A_1 as a natural ester concentrate with that of animals fed vitamin A_2 (1 gm. of oil solution with E (1 per cent, 1 cm.) (352 mμ) = 5) as a concentrate from *Stizostedion vitreum*. The natural ester concentrate of vitamin A_1 at this level depressed growth only slightly below normal, whereas the vitamin A_2 oil proved quite toxic, repressing growth to about one-sixth normal. However, it is by no means certain that this repression of growth is caused by the vitamin fraction of the oil.

DISCUSSION

Calculations from Optical Data—The amount of vitamin A_1 present in our vitamin A_2 concentrate was estimated. According to the experience of Wald (8), a number of vitamin A_1 preparations containing no vitamin A_2 possessed extinctions at 696 mμ with values 0.08 (average) of those at 618 mμ. Vitamin A_2 preparations which, in his opinion, contained no vitamin A_1, yielded bands possessing extinctions at 618 mμ with values 0.39 (average) of those at 696 mμ. According to these data of Wald, our vitamin A_2 concentrate would contain slightly less than zero amount of vitamin A_1. In our recent experience, we have found that pure vitamin A_1 preparations have extinctions at 695 mμ with values 0.05 of those at 620 mμ, while the purest vitamin A_2 preparations have extinctions at 620 mμ with values 0.33 of those at 695 mμ. It is evident that by our criteria the vitamin A_2 preparation described in this paper contains no vitamin A_1 and that the biological potency of the preparation was exerted by the vitamin A_2.

Lederer and Rathman (4) assume that vitamin A_2 preparations which contain no vitamin A_1 have extinctions at 620 mμ with values of 0.12 to 0.16 of those at 695 mμ. Karrer *et al.* (5) assume that pure vitamin A_2 preparations will have no extinction at 620 mμ. If Karrer is correct in his assumption, a considerable portion of the biological potency of our vitamin A_2 concentrate would be due to vitamin A_1. In our experience, a vitamin A_1 preparation will have a biological potency of about 750 U.S.P. units for each unit of value of E (1 per cent, 1 cm.) (620 mμ). Therefore, the biological potency due to vitamin A_1, if we assume that the extinction at 620 mμ was due to only vitamin A_1, would be 35,000 units per gm. This is a substantial amount of the total potency of 47,500 units per gm. found by bioassay. It is understandable why Karrer states that vitamin A_2 has little or no vitamin A activity as long as he assumes no 620 mμ absorption of the vitamin A_2-antimony trichloride complex.

We feel that the vitamin A_2-antimony trichloride band does have appreciable absorption at 620 mμ. It is interesting to note that Wald and ourselves are the only investigators in this field who have obtained their data with the help of a recording spectrophotometer which quickly and clearly gives the whole absorption curve for the antimony trichloride product.

Since there are no known methods for completely separating vitamin A_1 from vitamin A_2, it is hard to settle the disagreement about the relative amounts of the two vitamins present, as the methods of calculation differ. Embree and Shantz (9) found that anhydro vitamin A_1, however, can be readily separated from anhydro vitamin A_2 by chromatographic adsorption. A chromatographically pure concentrate of anhydro vitamin A_2 was prepared and found to have a ratio of E (695 mμ) to E (620 mμ) of 3.05 (Fig. 2). This, in our opinion, is confirmation of our contention that for vitamin A_2 the ratio of E (695 mμ) to E (620 mμ) is about 3.0.

SUMMARY

Liver oil concentrates obtained from *Esox lucius* have been examined physically, chemically, and biologically for vitamin A. Only vitamin A_2 is present. This possesses considerable vitamin A potency, as shown by the results of the usual bioassay technique on depleted rats. Furthermore, all of the vitamin A stored in the livers of these experimental animals is vitamin A_2.

The antimony trichloride reaction products of vitamin A_2 and anhydro vitamin A_2 have a value of about 3.0 for the ratio of E (695 mμ) to E (620 mμ).

BIBLIOGRAPHY

1. Lederer, E., Rosanova, V., Gillam, A. E., and Heilbron, I. M., *Nature*, **140**, 233 (1937).
2. Edisbury, J. R., Morton, R. A., and Simpkins, G. W., *Nature*, **140**, 234 (1937).
3. Gillam, A. E., Heilbron, I. M., Jones, W. E., and Lederer, E., *Biochem. J.*, **32**, 405 (1938).
4. Lederer, E., and Rathman, F. H., *Biochem. J.*, **32**, 1252 (1938).
5. Karrer, P., Geiger, A., and Bretscher, E., *Helv. chim. acta*, **24**, 161E (1941).
6. Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, **64**, 2411 (1942).
7. The pharmacopoeia of the United States of America, twelfth revision, Easton (1942).
8. Wald, G., *J. Gen. Physiol.*, **22**, 391 (1939).
9. Embree, N. D., and Shantz, E. M , *J Biol. Chem.*, **132**, 619 (1940).

THE COMPOSITION OF SWEAT, WITH SPECIAL REFERENCE TO THE VITAMINS*

By OLAF MICKELSEN and ANCEL KEYS

(From the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis)

(Received for publication, May 28, 1943)

Knowledge as to the composition of sweat is as yet fragmentary and many points are subject to dispute. Reasons for this are numerous, some of the more obvious being the difficulty of making satisfactory collections, variation in composition of sweat elaborated by different regions of the body, and the fact that exploratory work cannot be done on animals. The subject assumes considerable importance, however, when it is realized that in the tropics and hot deserts sweat production may amount to 5 to 12 liters a day (Dill (8) and Dill *et al.* (9)) and that this sweat is by no means a simple excretion of a dilute salt solution. If vitamins are lost in sweat, as has been claimed recently, there is a possible basis of support for such claims as that of Mills (22) that vitamin requirements are elevated in hot climates.

This paper is a report on the composition of sweat with special reference to vitamins and some organic substances and to variations in composition of sweat collected from different parts of the body and at different times. Great care was taken in the sampling procedures and in the analytical methods used.

Subjects and Experimental Conditions

All subjects were "normal" young men who were free from known physical defects. Some of the subjects were soldiers of the 710th M. P. Battalion; others were college students. All were moderately active physically but none was in real athletic training.

All of the studies were carried out in the late fall, winter, and early spring, so that the subjects were not acclimatized at the outset. The subjects were studied, and in some cases housed continuously for some days at a time, in an experimental suite with controlled temperature and

* The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Minnesota. Important financial assistance was also provided by the Nutrition Foundation, Inc., by the Corn Industries Research Foundation, by Swift and Company, Chicago, by the National Cane Sugar Refiners' Association, and by the National Confectioners' Association. We are grateful to Merck and Company, Inc., for supplies of pure vitamins.

humidity. The majority of the sweat collections were carried out in an environmental temperature of 49° and humidity of about 25 per cent relative saturation. In some cases an equivalent "effective" temperature (Houghton and Yagloglou (15)) was achieved with lower temperatures and higher humidities. Collections were made on resting subjects and on subjects during or immediately following moderately hard work on the motor-driven treadmill. The conditions were generally such as to provoke an average sweat loss of 5 to 8 liters a day. Water drinking was allowed *ad libitum*. Diet was controlled to insure that standard conditions prevailed. In some cases salt or vitamin intakes were restricted or augmented above normal. In all cases salt, water, and vitamin intakes were measured.

Analytical Methods

Chlorides were estimated by the mercurimetric titration of Cavett and Holdridge (3) and by the Volhard method of Keys (16). Glucose was estimated by the method of Folin and Wu. Lactic acid was determined by the method of Friedemann, Cotonio, and Shaffer as modified by Edwards (11). Urea analyses were made by a modified Folin-Svedberg method. Ammonia was estimated by the same procedure with omission of urease. Creatinine was determined by the method of Folin and Wu modified for use with the photoelectric colorimeter.

Ascorbic acid was estimated by a modification of the Mindlin and Butler technique (23). The analysis for thiamine involved a direct oxidation of the vitamin by means of alkaline potassium ferricyanide. The thiochrome was extracted with isobutyl alcohol and then read in a photofluorometer. Blanks were always run on the same samples without the addition of potassium ferricyanide.

Riboflavin analyses were made both by the microbiological method of Snell and Strong (29) and by the fluorometric method of Conner and Straub (5). Nicotinic acid was determined by the microbiological assay of Snell and Wright (30).

Methods of Sweat Collection

In all cases the skin from which sweat was to be collected was carefully washed with soap and water, rinsed with distilled water, and then dried. Profuse sweating was then induced, the first sweat being quickly sponged off with distilled water and the skin dried before collections were started for analysis.

Sweat from the arm and hand was collected in arm length, duprene gauntlets, without fingers, attached to a 25 cc. Florence flask. The flask was suspended in a bathing cap filled with cracked ice, so that the sweat was chilled and kept cold during the collection period. The top of the gauntlet

was tied snugly around the upper part of the biceps. In some experiments arm length, neoprene gauntlets, complete with fingers, were used.

In the first experiments with sweat from different parts of the body the room humidity was elevated, profuse sweating induced, and sweat was collected by scraping the skin with a small beaker or pus basin. Just before each sample was taken in this way the skin was quickly sponged with distilled water and dried. This procedure was relatively satisfactory for the back and chest when sweat production was so profuse that samples were obtained within 2 or 3 seconds after the skin was prepared.

In the later experiments sweat was collected from different parts of the body in gauze bandages covered externally with dental rubber dam. The sweat was extracted from the bandage and rubber dam by a measured amount of distilled water. Both gauze and rubber dam were weighed dry and the combined sweat-bandage-rubber dam was weighed to measure the total sweat collected. This method proved very satisfactory for sweat collection from the face, axilla, thigh, and various parts of the torso, though some practice is needed to apply and remove the bandages properly.

In the past, much work on sweat composition has been done with sweat collected from the hand in an ordinary surgical rubber glove (Dill (8)). In order to compare such sweat with that of the entire body the subjects were clad in a full length union suit and heavy socks and wore the rubber gloves while walking briefly on the treadmill. The skin was previously prepared as usual and the garments had been carefully washed in distilled water, dried, and weighed. No sweat as such was lost from the system, body plus garments. At the end of the period of sweating the subject stood in an enameled basin into which the garments were removed and the body rinsed with distilled water. The garments plus washings were weighed. The garments were then extracted with the distilled water and analysis for chloride was carried out on the diluted sweat and washings. Glove sweat was analyzed directly. Before and after the sweat collection the nude subject was weighed to ± 6 gm. The total sweat production was large enough so that this weighing procedure allowed estimation of the volume with an error of about 1 per cent.

Results

Ascorbic Acid—A large number of recovery experiments showed that sweat collection for ascorbic acid estimation is very satisfactorily accomplished with the duprene gauntlets in 15 minutes at 49°. Vitamin C destruction in the collection of sweat for analysis is negligible when the arrangement with ice-cold collecting vessels is used.

With subjects on ordinary diets the concentration of ascorbic acid was always very low, ranging from 0 to 0.18 mg. per 100 cc., with most of the

values below 0.10 mg. Experiments were then carried out to discover what relation, if any, may exist between vitamin C intake and the concentration of the vitamin in the sweat. In the first series we studied twelve subjects who were maintained on known fixed intakes for 4 to 7 days at a time. The two levels of vitamin C intake studied were 25 and 525 mg. per day. The results, summarized in Table I, clearly show that the ascorbic acid concentration in the sweat is not only low but does not reflect a great difference in dietary intake.

TABLE I

Concentration of Vitamin C in Sweat Collected from Men on Different Levels of Dietary Intake of Vitamin

The values are expressed in mg. per 100 cc.

Intake	No. of samples	Vitamin C, mean	Standard deviation	Range
mg. per day				
525	30	0.0590	±0.0467	0–0.16
25	28	0.0603	±0.0559	0–0.18

TABLE II

Concentration of Vitamin C in Sweat before and at 2 and 6 Hours after Ingestion of 50 Mg. of Pure Ascorbic Acid

The values are expressed in mg. per 100 cc.

Subject	Before	2 hrs. after	6 hrs. after
N. O.	0.039	0.026	0.033
K. O.	0.027	0.032	0.038
K. E.	0.025	0.032	0.025
M. E.	0.026	0.017	0.050
R. O.	0.017	0.030	0.027
G. E.	0.032	0.027	0.039
Mean	0.028	0.027	0.035

In another series six men were studied before and at 2 and at 6 hours after ingesting 50 mg. of pure ascorbic acid. The results are given in Table II, which shows that any effect of this dosage is very slight at most. The appearance of an average increase in concentration of ascorbic acid in the sweat 6 hours after the 50 mg. dose is chiefly due to the result on subject M. E.

Incidentally, it may be noticed that the values in Table II are considerably lower than those in Table I. It is believed that the lower values are more nearly correct. With our most recent analytical technique in which

large samples are used we have found the average ascorbic acid concentration in the sweat of twelve young men on ordinary college diets to average 0.033 mg. per 100 cc., standard deviation = ±0.0064.

The concentration of ascorbic acid in the sweat is independent of that in the plasma, as might be inferred from the results cited above. In subjects with values for plasma vitamin C of from 0.31 to 1.41 mg. per 100 cc. the sweat concentrations were entirely independent.

It should be noted that the decolorization of 2,6-dichlorophenol indophenol is not entirely specific for ascorbic acid. We can conclude that the concentration of ascorbic acid in the sweat is not greater than indicated above. It is clear, therefore, that even in the most extreme conditions, when the sweat volume may reach 10 liters per day, the maximal loss of ascorbic acid is less than 10 mg. Actually, the maximal losses we have calculated from studies in the laboratory and in extreme conditions in desert maneuvers with the United States Army would amount to 4 mg. per day or less. The physiological rôle of ascorbic acid in resistance to high temperatures will be discussed in a separate paper.

Vitamins of the B Complex—Sweat contains a large amount of fluorescent substance but this is mostly not thiamine or riboflavin. When controls are run by omitting potassium ferricyanide and these are used in the calculation of the true thiamine content from the thiochrome procedure, it appears that the thiamine concentration of sweat is exceedingly small and accordingly very difficult to estimate accurately. In a series of twelve separate estimations of thiamine in sweat, values have ranged from 0.00 to 0.6 γ per 100 cc. The average is of the order of 0.15 γ per 100 cc. and even this may be an overestimate. If we accept the highest possible values, it appears that maximal loss of thiamine in sweat would be less than 50 γ per day; actually we believe the true maximal loss is considerably less than this amount.

Riboflavin similarly is found in sweat only in vanishingly small amounts. In order to achieve certainty on this point much care was taken in collection and large pooled samples were concentrated by evaporation in a vacuum at low temperature. Microbiological assays were carried out on the concentrated material and indicated about 0.5 γ of riboflavin per 100 cc. Accordingly, the maximal sweat loss of this vitamin is no greater than in the case of thiamine.

Nicotinic acid in sweat was measured in pooled samples and averaged about 100 γ per 100 cc. Both chemical and microbiological methods gave results in agreement.

Different Parts of Body—It is well known that lactic acid and urea as well as chloride occur in sweat in significant amounts. Accordingly, it was considered that the estimation of these substances in sweat collected simul-

taneously from different parts of the body would help to describe the constancy or variability of the total sweat concentration produced in different regions as well as the concentration of the individual substances.

In the first series of experiments sweat was collected from the arm by the gauntlet and from the back and abdomen by rapidly scraping the free flowing sweat as previously described. The temperature was 38° and the humidity was 65 per cent relative saturation. Collections and analyses were carried out so as to minimize evaporation and destruction of lactate. The results of these analyses on sweat from six subjects are summarized in Table III.

TABLE III

Composition of Sweat Collected Simultaneously from Different Parts of Body

In each case the values for the back and for abdominal sweat are expressed as percentages of the value of the same substance in the arm sweat collected at the same time from the same subject.

Subject	Source	Chloride	Lactate	Urea
M. E.	Back	128	94	112
"	Abdomen	147	89	123
P. A.	Back	119	70	81
"	Abdomen	143	72	83
M. J.	Back	140	84	85
"	Abdomen	133	70	100
D. U.	Back	184	92	75
"	Abdomen	133	66	82
M. A.	Back	176	41	78
"	Abdomen	162	44	85
P. E.	Back	154	92	68
"	Abdomen	143	108	80
Mean	Back	150	79	83
"	Abdomen	144	75	92

There are very marked variations in the concentrations of these substances in the sweat collected from different parts of the body and these cannot be explained as the result of different rates of evaporative water loss from the sweat in different regions. In general the arm sweat was least concentrated in chloride but most concentrated in lactate and tended to be most concentrated in urea.

Equally marked differences were obtained by the method in which sweat was collected in gauze bandages, when evaporative losses were entirely excluded. In this second series of experiments creatinine and uric acid were added to the analytical battery. Typical results from two experi-

ments on one subject, carried out 2 weeks apart, are summarized in Table IV.

There is some evidence that the variation in concentration of lactate, urea, creatinine, and uric acid in sweat from different parts of the body is reduced in the subject acclimatized to the heat. Whether this is a result of profuse sweating continued for some days or some other factor involved in acclimatization cannot be stated. Illustrative results are tabulated in Table V.

TABLE IV

Composition of Sweat Collected Simultaneously from Different Parts of Body of Unacclimatized Subject

The results of two experiments, performed 2 weeks apart, are tabulated here. Chloride values are in mM per liter; all other values are in mg. per 100 cc.

Region	Experiment 1					Experiment 2				
	Cl	Lactate	Urea N	Creatinine	Uric acid	Cl	Lactate	Urea N	Creatinine	Uric acid
Torso.	55	204	38	0.70	0.23	69	125	34	0.66	0.32
Face	101	157	51	0.90	0.95	98	128	36	0.74	1.15
Thigh..	75	296	60	0.75	0.35	82	177	44	1.06	0.10
Axilla	114	186	39	0.90	1.42	56	59	29	0.63	0.97
Arm.	55	250	41	0.85		60	228	40	1.90	0.04

TABLE V

Composition of Sweat from Different Parts of Body before and after Acclimatization to High Temperature

Chloride values are in mM per liter; all other values are in mg. per 100 cc.

Region	Before acclimatization					After acclimatization				
	Cl	Lactate	Urea N	Creatinine	Uric acid	Cl	Lactate	Urea N	Creatinine	Uric acid
Thigh .	57	382	39	0.76	1.55	18	123	29	0.82	0.25
Torso.. .	43	121	15	0.33	0.18	16	104	23	0.58	0.13
Face. .. .	57	144	30	0.71	0.39	32	105	30	0.86	0.20
Arm. .	29	174	38	0.67	0.39	36	161	34	0.66	0.40

Chlorides—The variability of chloride concentration in the sweat has been remarked by all investigators. It might be thought that the sweat chloride reflects, in an exaggerated manner, changes in the plasma chloride. This is not normally the case, as is shown in Fig. 1. There was no trace of a relation between the chloride concentration in the blood plasma and in the arm plus hand sweat in eighteen normal young men on either the 1st or the 2nd day of exposure to the heat. These men were on a diet slightly restricted in chloride. The chloride concentration in both fluids

averaged somewhat less on the 2nd day than on the 1st but these differences were statistically insignificant.

Earlier in this paper we indicated important differences in the concentration of sweat collected from different parts of the body. The inference is that it may not be possible to calculate the total sweat loss of chloride from the analysis of a "sample." Such calculations have been made

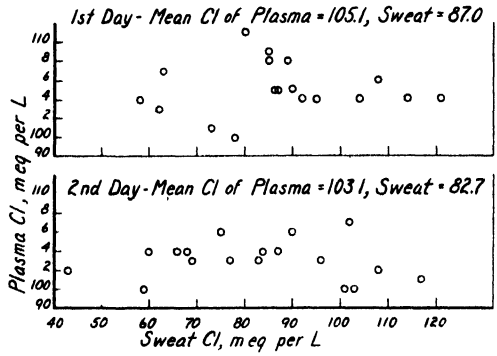


FIG. 1. The relation between the concentrations of plasma and sweat chloride during the first 2 days in the heat.

TABLE VI

Comparison of Average Chloride Concentration of Total Body Sweat with Chloride Concentration in Sweat Collected Simultaneously from Hand

Chloride concentrations are in milliequivalents per liter. "Weight loss, corrected" is the gross body weight loss corrected for R.Q. and insensible perspiration. The sweat collection periods ranged from 30 to 45 minutes each.

Subject	Date	Hand [Cl]	Body weight loss	Weight loss, corrected	Total Cl loss	Body [Cl]	Hand [Cl] as per cent of body [Cl]
			gm.		*mg*		
L.	Feb. 23	62.0	266	237	10.7	45.1	138
"	" 27	57.2	650	629	24.3	38.6	148
S.	Mar. 4	83.2	761	730	39.1	53.6	155
"	" 4	65.8	713	682	26.1	38.3	172

chiefly from hand sweat collected in a rubber glove. The acceptability of this procedure was carefully checked in four experiments in which hand sweat was collected simultaneously with that of the rest of the body. The results are given in Table VI. It is clear that very large errors would result from the calculation of total body losses of chloride from the concentration of hand sweat and the weight loss.

DISCUSSION

The composition of sweat is of interest because of the possible effect of sweat loss on the requirements of the body for salt and vitamins in hot environments. In the case of salt (chloride) the loss may be large and its quantitative evaluation is important. However, it appears that such evaluation cannot be made accurately from samples taken from any one part of the body. In general, the total salt loss calculated from the concentration of sweat collected in a glove is considerably greater than the true salt loss from the whole body; this is well shown in the present study. The relatively low salt concentration of total body sweat is notable in the measurements of Hancock, Whitehouse, and Haldane (13), of Dill *et al.* (10), and of Lee *et al.* (19). It should be noted that the loss of water and salt through the skin is not independent of osmotic forces (Whitehouse, Hancock, and Haldane (32)), and it must be expected that the sweat concentration will vary according to whether the skin is immersed in sweat (as in a glove) or encased in salt (as in complete evaporation).

We have no ready explanation for the seemingly independent variation in the concentrations of different constituents in the sweat collected from different parts of the body. However, it should be remembered that the total sweat is the product of three different sources: (1) secretion of the eccrine glands, (2) secretion of the apocrine glands, and (3) presumably passive diffusion of water and possibly other constituents in the "insensible perspiration." The concentrations of the several constituents differ in the "sweat" from the different sources and the relative amounts of the several sweats produced in different regions must vary according to the anatomy of the local skin (*cf.* Kuno (18)). The subject of chloride economy under different conditions will be discussed in a separate contribution.

Numerous statements are appearing to the effect that human vitamin requirements are increased in hot environments (*cf.* Mills (22)). Most of the factual reports concern ascorbic acid losses in sweat. Cornbleet, Klein, and Pace (7) and Bernstein (1) reported vitamin C concentrations in sweat of the order of 0.5 to 1.0 mg. per 100 cc. Such values could mean important losses in heavy sweating. However, other analyses indicate much smaller concentrations (Zselyonka and Nánássy-Mégay (34), Wright and MacLenathen (33), Hardt and Still (14)). The present results are still smaller and indicate that ascorbic acid loss in sweat is negligible. The higher values reported earlier probably reflect the fact that the analytical methods used are not very specific and other reducing substances readily enter as contaminants. We have observed this to be the case with some types of rubber gloves.

Hardt and Still (14) have suggested that thiamine losses in sweat may "cause serious physiological consequences." Their values for thiamine

concentration in sweat must be ascribed to analytical error; we found that the more exacting were our methods the nearer our results were to the zero thiamine level. Slater (28) has reported similarly low values for thiamine in sweat.

Many workers have reported significant amounts of glucose in sweat, 5 to 20 mg. or more per 100 cc. (*e.g.* Borchardt (2), Sylvers *et al.* (31), McSwiney (20)). We obtained similar values by the use of the ordinary methods. However, sweat contains a number of non-glucose reducing substances and the ordinary methods are not specific enough to allow conclusions as to the true glucose content of sweat. Schulze (26) and Schulze and Kunz (27) have shown that practically none of the substance in sweat recorded as glucose by the Hagedorn-Jensen method will form a phenylglucose osazone. The statement of Engelhardt (12) that sweat of diabetic patients may exceed 150 or 200 mg. of glucose per 100 cc. is certainly erroneous.

The majority of the constituents in sweat are more dilute than in the blood plasma or interstitial fluid. Lactic acid, or lactate as it exists in sweat, is a notable exception. Values as high as 450 mg. of lactic acid per 100 cc. have been reported in the sweat of resting subjects (Pemberton (24)), but the best methods indicate that the values are usually below 100 mg. per 100 cc. unless the blood lactate level is elevated. The values recorded here reflect only moderate elevation in the blood lactate. Kosiakina and Krestownikoff (17) reported values as high as 1765 mg. per 100 cc. of sweat obtained in a marathon race.

Urea is also more concentrated in sweat than in the blood. In the present study thirty-eight samples of arm plus hand sweat averaged 67 mg. of urea per 100 cc., with a range of 23 to 156 mg. The bloods drawn at the same time averaged 18.1 mg. per 100 cc., with a range of 8.2 to 24.4 mg. These sweat values for urea are higher than those reported by McSwiney (20), Mezinesco (21), Chopra *et al.* (4), and Robinson *et al.* (25), but it may be noted that urea is not very stable in sweat and special precautions are needed to prevent its loss in the collected samples. The urease method used here is highly specific and was applied with great care; so considerable confidence is placed in the results.

It should be clear that sweat secretion is at least as complicated as urine secretion and sweat is, in its way, as complex as urine. But in the case of urine formation some regularity is apparent and the relations between the urine and the blood pressure are at least capable of partial definition. As yet few guiding principles for the quantitative appearance of various substances in sweat are discernible. Such substances as glucose, thiamine, riboflavin, and androgens (Cornbleet and Barnes (6)) are almost wholly excluded; phosphates, uric acid, and ascorbic acid appear in very small

amounts; and ammonia, urea, and lactate are greatly concentrated. Chloride ranges from twice the concentration in the plasma to perhaps one-fifth the plasma level. None of these substances reflects the blood concentration of the same substances unless the blood levels are markedly abnormal.

SUMMARY

1. Sweat was collected from various parts of the body of normal young men in rest and in moderate work. In most cases the temperature was 49°, the humidity 25 per cent relative saturation, but in some experiments humidities over 65 per cent relative saturation were maintained at an air temperature of 37–38°.

2. The concentration of ascorbic acid in sweat is generally less than 0.1 mg. per 100 cc., the true average being of the order of 0.03 mg. per 100 cc. The concentration is independent of the dietary intake of the vitamin.

3. The concentration of thiamine in sweat is of the order of 0.2 γ or less per 100 cc. Riboflavin concentration is of the order of 0.5 γ or less per 100 cc. of sweat.

4. The concentration of nicotinic acid or its biological equivalent in sweat is of the order of 0.1 mg. per 100 cc.

5. Lactate, urea, and ammonia are much more concentrated in the sweat than in the blood.

6. Sweat samples collected simultaneously from the hand, arm plus hand, face, torso, and thigh showed marked differences in total concentration and in concentration of individual constituents including chloride, lactate, urea, creatinine, and uric acid.

7. Hand sweat is more concentrated in chloride, by 30 to 70 per cent, than the total body sweat formed at the same time.

8. The concentration of chloride in the sweat is highly variable but it is independent of moderate variations in the concentration of chloride in the blood plasma.

9. Under conditions of continued profuse sweating losses of ascorbic acid, thiamine, and riboflavin are negligible from the standpoint of vitamin nutrition. The sweat loss of nicotinic acid under these conditions could be significant, however.

It is a pleasure to record our obligations to Colonel H. J. Keeley, Commanding Officer, Fort Snelling, to Lieutenant-Colonel J. J. Shy, Commanding Officer, 710th Military Police Battalion, and to the men of the 710th Military Police Battalion who served as subjects. This work would have been impossible without the generous help of the staff of the Laboratory of Physiological Hygiene, especially Mr. Howard Condiff and Miss Doris

Doeden, Assistant Chemists, and Miss Angie Mae Sturgeon, Head Technologist. We are greatly indebted to Dr. J. S. Andrews of General Mills, Inc., for analyses for nicotinic acid and riboflavin.

BIBLIOGRAPHY

1. Bernstein, R. E., *Nature*, **140**, 684 (1937).
2. Borchardt, W., *Arch. ges. Physiol.*, **214**, 169 (1926).
3. Cavett, J. W., and Holdridge, C. E., *J. Lab. and Clin. Med.*, **18**, 944 (1932).
4. Chopra, R. N., Roy, A. C., and Biswas, H. K., *Indian J. Med. Research*, **27**, 931 (1940).
5. Conner, R. T., and Straub, G. J., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 385 (1941).
6. Cornbleet, T., and Barnes, B., *Arch. Dermat. and Syphilol.*, **41**, 654 (1940).
7. Cornbleet, T., Klein, R. J., and Pace, E. R., *Arch. Dermat. and Syphilol.*, **34**, 253 (1936).
8. Dill, D. B., Life, heat and altitude, Cambridge (1938).
9. Dill, D. B., Hall, F. G., and Edwards, H. T., *Am. J. Physiol.*, **123**, 412 (1938).
10. Dill, D. B., Jones, B. F., Edwards, H. T., and Oberg, S. A., *J. Biol. Chem.*, **100**, 755 (1933).
11. Edwards, H. T., *J. Biol. Chem.*, **125**, 571 (1938).
12. Engelhardt, W., *Münch. med. Woch.*, **80**, 2037 (1933).
13. Hancock, W., Whitehouse, A. G. R., and Haldane, J. S., *Proc. Roy. Soc. London, Series B*, **105**, 43 (1929).
14. Hardt, L. L., and Still, E. W., *Proc. Soc. Exp. Biol. and Med.*, **48**, 704 (1941).
15. Houghton, F. C., and Yagloglou, C. P., *Tr. Am. Soc. Heat. and Vent. Eng.*, **29**, 163 (1923).
16. Keys, A., *J. Biol. Chem.*, **119**, 389 (1937).
17. Kosiakina, A. F., and Krestownikoff, A. N., *Arbeitsphysiologie*, **2**, 421 (1930).
18. Kuno, Y., The physiology of human perspiration, London (1934).
19. Lee, D. H. K., Murray, R. E., Simmonds, W. J., and Atherton, R. G., *Med. J. Australia*, **28**, 249 (1941).
20. McSwiney, B. A., *Proc. Roy. Soc. Med.*, **27**, 839 (1934).
21. Mezinesco, M. D., *Bull. Soc. chim. biol.*, **20**, 39 (1938).
22. Mills, C. A., Climate makes the man, New York, 35 (1942).
23. Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, **122**, 673 (1937–38).
24. Pemberton, R., Arthritis and rheumatoid conditions, London, 113 (1929).
25. Robinson, S., Dill, D. B., Wilson, J. W., and Nielsen, M., *Am. J. Trop. Med.*, **21**, 261 (1941).
26. Schulze, W., *Arch. Dermat. u. Syph.*, **181**, 471 (1940).
27. Schulze, W., and Kunz, K., *Arch. Dermat. u. Syph.*, **181**, 486 (1940).
28. Slater, E. C., *Australian J. Sc.*, **4**, 136 (1942).
29. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
30. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
31. Sylvers, S., Forster, W., and Talbert, G. A., *Am. J. Physiol.*, **84**, 577 (1928).
32. Whitehouse, A. G. R., Hancock, W., and Haldane, J. S., *Proc. Roy. Soc. London, Series B*, **111**, 412 (1932).
33. Wright, I. S., and MacLenathen, E., *J. Lab. and Clin. Med.*, **24**, 804 (1939).
34. Zselyonka, L., and Nánássy-Mégay, K., *Orvosi Hetilap*, **81**, 800 (1937); *Chem. Abst.*, **31**, 7098 (1937).

SOY BEAN LIPOXIDASE*

By A. K. BALLS, BERNARD AXELROD, and MARIAN W. KIES

(From the Enzyme Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, May 20, 1943)

Acceleration of the oxidation of xanthophyll and similar substances to colorless products has long been recognized as a property of soy bean meal and used in the bleaching of bread dough. Hauge and Aitkenhead (1) reported a carotene-destroying factor in alfalfa, and later (Hauge (2)) a similar enzyme in potato juice. André and Hou (3) showed that the fat of soy beans and of certain true beans could be oxidized by a catalyst present in the beans. Craig (4), using the Warburg technique, demonstrated the oxidation of a variety of fats by means of protein constituents of *Lupinus albus*. Sumner and Dounce (5) showed that an enzyme, "carotene oxidase," existed in aqueous extracts of soy beans. Its action was dependent upon the presence of unsaturated fats or fatty acids, thus connecting the oxidation of carotene and fat. Unsaturated fats were found to increase in peroxide number in the presence of soy bean meal (6).

The evidence presented by Sumner and Sumner (6) and by Tauber (7) greatly favors the view that the oxidation of carotene occurs at the expense of oxidized fat, and that the oxidation of carotene is not enzymatically catalyzed by this system. Accordingly the enzyme is now regarded as a lipoxidase. However, fat that has been previously oxidized by the enzyme does not spontaneously oxidize carotene added to it thereafter. Thus, as stated by Strain (8) and R. J. Sumner (9), not the stable end-product of fat oxidation by the enzyme but rather an intermediate or nascent product of that process is responsible for the oxidation of carotene.

Süllmann (10) noted that partial inactivation of the enzyme extracted from soy beans could be caused by prolonged dialysis, owing perhaps to the removal of an activating substance. He also found that while linoleic and linolenic acids used oxygen equivalent to one and two double bonds respectively, oleic and ricinoleic acids consumed only about a tenth as much oxygen as required for one double bond.

The system leads to the loss of carotene, vitamin A, ascorbic acid, and chlorophyll. It also accelerates the initial reactions producing "rancidity."

* Enzyme Research Laboratory Contribution No. 85. This work was done on Bankhead-Jones funds. Part of this work is from a thesis submitted to Georgetown University by Bernard Axelrod in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Its possible activity should therefore be considered in any effort to handle foodstuffs. Fortunately, this enzyme appears to be restricted in its occurrence in the plant world. Potatoes, asparagus, and wheat germ (11) contain small amounts; legumes, such as alfalfa, peas, peanuts, and particularly soy beans contain a considerable quantity. The enzyme is moreover easily destroyed by heat or acid. Commercial soy bean meal, a by-product of the preparation of soy bean oil, contains little or no lipoxidase.

In the work presented here, a quick and easy method for the measurement of the enzyme has been described, based on the rate at which a carotene suspension is bleached. By the application of this method a notable purification of the enzyme prepared from soy bean meal has been achieved.[1]

A study of the purified enzyme has led to the finding that crude preparations are accompanied by a thermostable substance that increases the catalyzed rate of oxidation of fats as well as of carotene and other secondary substrates. An approximate method for its estimation has been developed, by the aid of which considerable purification of the active substance was achieved. The purified material is free of ash and consists largely of one or more peptides, but as the active substance has not yet been crystallized these may be merely impurities.

There is some evidence that the action of the accelerating substance is on the fat, not on the enzyme. It may not, therefore, strictly speaking, be an "activator." However, since this term has so frequently been employed for any substance that hastens an enzyme action, it will be used here to refer to the accelerating substance in soy beans.

Previous workers (6), using less purified enzyme preparations, found that the rate of oxidation as measured by the coupled destruction of carotene varied with the fat present in such a way that, for most rapid oxidation, there was a sharply defined optimum quantity of fat. It is now apparent that this optimum was observed, because for larger doses of fat there was an insufficient supply of activator; for when both fat and activator are increased, the rate of oxidation with a given amount of enzyme becomes nearly constant over a considerable range of fat concentrations.

Among the many inhibitors of lipoxidase action, crystalline purothionin (13) is specially potent. The inhibition is decreased by increasing the amount of fat and in other ways resembles in reverse the action of the activator. Since purothionin appears to inhibit because of surface effects in the fat suspension, it is possible that the activator accelerates for similar reasons.

The specificity of the fat oxidase has been dealt with by several workers

[1] Another method of determining lipoxidase activity based on carotene destruction has recently been published by Reiser and Fraps (12). The method presented here is much less cumbersome, since it obviates the necessity of extracting the carotene from the reaction mixture.

(8, 10, 14, 15). Opinion is divided as to the susceptibility of oleic acid to oxidation. In our experiments, pure oleic acid was found not to be oxidized. A study of the behavior of exceptionally pure specimens of fats and fatty acids showed that out of a considerable number only linoleic, linolenic, and arachidonic acids (or esters) were attacked by lipoxidase. These acids were the only members of the "essential" fatty acids (16) available to us, and they were the only acids found to be oxidized. It is therefore probable that the direct action of lipoxidase is limited in nature to the biologically essential fatty acids.

This probability leads naturally to the suggestion that the essential fatty acids serve as intermediate catalysts in a still undescribed system of tissue oxidation. Attempts to find such a cyclic series of reactions have been hampered by the artificial conditions under which the enzyme has had to be handled, and no good evidence has been found yet to support what appears to be a very likely hypothesis.

Purification of Soy Bean Lipoxidase—500 gm. of finely ground soy bean meal (Tokyo variety), which had been freed of fat by exhaustive extraction with petroleum ether, were suspended in 10 liters of cold water. The suspension was cooled to 7°. Enough M acetic acid was added to adjust the pH to 5.1 to 5.2 and the solution was immediately filtered. The total filtrate (8300 ml.) was brought to pH 6.8 with M NaOH. Solid ammonium sulfate was added to 0.5 saturation. Without delay, M NaOH was added until the pH of the suspension was readjusted to 6.8. The precipitate was collected by centrifugation and dissolved in 500 ml. of H_2O. On standing, this solution deposited a partly crystalline sludge which possessed insignificant enzyme activity and was discarded. Ammonium sulfate was then added to 0.3 saturation. The addition was followed by immediate adjustment to pH 6.8. The precipitate was discarded and the supernatant liquid made 0.4 saturated. This precipitate was refractionated with ammonium sulfate at 0.35 saturation to remove the less soluble portion. The combined supernatant liquids were made 0.45 saturated and the highly active precipitate so obtained was dissolved in water. The solution was dialyzed in cellophane tubing against cold running tap water until the test for sulfate was negative. The solution was next treated with a 2 per cent neutralized solution of crystalline purothionin hydrochloride (13) until no more precipitation occurred. Considerable inactive material was removed by this means. The supernatant was then subjected to further fractionation with ammonium sulfate. Table I shows the result of these final steps. The precipitates obtained at 0.47 and 0.5 saturation were combined and served as the source of purified enzyme used in this work. The specific activity of this preparation, based on protein N, is 115 times that of a 2.5 per cent water extract of soy bean meal.

Assay of Lipoxidase with Carotene As Indicator—Crystalline carotene

(10 per cent α- and 90 per cent β-carotene) was purified by the method of Fraps and Kemmerer (17). A stock solution in 90 per cent acetone-10 per cent ethanol was prepared, containing 43 γ of carotene and 100 γ of ethyl linoleate per ml. 5 ml. of the stock solution were run into 52.5 ml. of phosphate buffer (50 ml. of H_2O and 2.5 ml. of 0.66 M buffer at pH 6.5). The tip of the pipette should be kept about 5 mm. above the surface of the liquid. Shaking should be avoided. The suspension is stable for 20 to 30 minutes, and is adversely affected by high room temperature and direct sunlight.[2] 10 ml. of the carotene-fat suspension were placed in a tube suitable for use in the Evelyn colorimeter. The enzyme to be assayed was pipetted into a 50 ml. Erlenmeyer flask and sufficient activator, or other desired material, and water added to give a final volume of 2 ml. The contents of the tube were poured into the flask, the two liquids mixed with a

TABLE I

Purification of Soy Bean Lipoxidase

Fraction	Total N	Activity, total units*	Units per mg N
	mg.		
Before treatment with purothionin. .	1310	368,000	280
After " " "	470	273,000	580
Ppt. at 0.43 saturation. . . .	87	36,400	420
" " 0.45 " . .	43.3	21,400	495
" " 0.47 "	76.2	48,500	635
" " 0.50 "	79.6	54,000	680
" " 0.60 "	52.7	18,200	345
" " 1.0 " . . .	3.57	0	

* Determined in the presence of an optimum amount of purified activator.

minimum of agitation, and the mixture poured back into the colorimeter tube. Colorimeter readings were made at 30, 45, or 60 seconds. The per cent of carotene oxidized at any time may be found from an appropriate calibration curve.

1 unit of lipoxidase activity is defined as that amount of enzyme that bleaches 30 per cent of the carotene-fat suspension in 30 seconds under the conditions described for the assay. The accuracy was greatest when 20 to 50 per cent of the carotene was destroyed in 30 seconds.

Fig. 1 relates units directly to galvanometer readings. It represents the readings obtained when a purified lipoxidase solution (containing sufficient activator) acted on the standard substrate suspension at 30, 45, and 60 second intervals.

[2] It has since been found that a more stable suspension results when the carotene solution is run into water and the buffer added just prior to the enzyme. This is our present procedure.

Measurement of Lipoxidase Action with Leuco Dyes—A number of leuco dyes are readily oxidized by the oxidase-fat system and a method for measuring the activity of the enzyme may be based on the rate of development of color. Such a scheme allows the use of so much indicator that the observed rate of reaction is practically independent of the concentration of indicator. This is not the case with the limited amount of carotene that can be used. Furthermore, a dye may be selected whose oxidation requires a known amount of oxygen. The experimental set-up is therefore more flexible and deserves future attention.

Leuco-*o*-chlorophenol indophenol was found to be a suitable dye for the purpose. The procedure followed that described in the preceding section dealing with the oxidation of carotene, except that the solution of fat con-

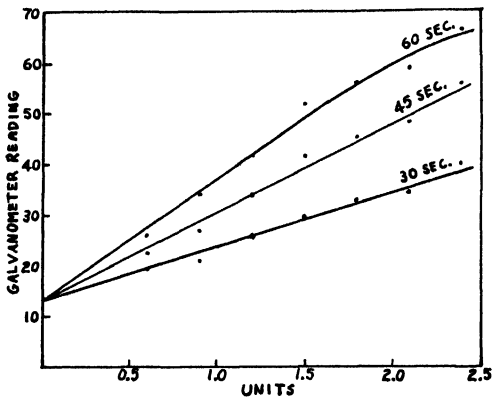


FIG. 1. Photometric determination of lipoxidase activity when carotene-ethyl linoleate is used as substrate.

tained no carotene, and the leuco dye, in aqueous solution, was mixed with the diluted phosphate buffer. An Evelyn Filter 635 was used.

Method of Estimating Peroxide Formed in the Fat—The method is essentially that described by Young, Vogt, and Nieuwland (18) for the estimation of organic peroxides, except that the concentration of sulfuric acid has been increased. A similar method has recently been recommended by Sumner (11) for assaying lipoxidase.

A substrate was prepared consisting of 50 ml. of water, 2.5 ml. of 0.66 M phosphate buffer (pH 6.5), and 5 ml. of acetone containing the desired lipid. 10 ml. of the substrate were placed in a 50 ml. flask. Another flask contained enzyme, activator, and enough water to make a total volume of 2 ml. The contents of the two flasks were carefully mixed. At desired intervals, 2 ml. portions of the reaction mixture were transferred to a colorim-

eter tube containing 10 ml. of the peroxide reagent.[3] The tubes were placed in water at 60° for 2 minutes and then cooled. The color values were read in an Evelyn colorimeter with Filter 540.

A preliminary attempt was made to adapt this method to the assay of lipoxidase. Ethyl linoleate, although useful in the carotene method, was not satisfactory here. Its relatively low solubility permitted the measurement of only a very restricted range of enzyme. Linoleic acid, used by Sumner, is not open to this objection, but commercial preparations are of too uncertain composition to warrant their use in a standard method.

It does appear, however, that peroxide may be slowly used up as well as formed during the reaction. In experiments run for a considerable time, the peroxide content of the fat was actually observed to decrease when crude activator was used (Fig. 4, Curve B′). Süllmann (19) has shown that carbonyl compounds may also be formed from fatty acids through the action of lipoxidase. The peroxide content of the fat cannot therefore be taken as a measure of total oxidation.

Preparation and Estimation of Activator—A crude preparation of activator suitable for many purposes was made by rubbing 5 gm. of fat-free soy bean meal in a mortar with 100 ml. of water and adding 2 ml. of M acetic acid. The suspension was heated to 95° for 10 minutes, filtered, and was neutralized just before use.

A convenient means of estimating the activator was to determine the time required to bleach half of the carotene in the standard assay system both in the presence and absence of activator. An amount of purified enzyme was chosen which without added activator destroyed half the carotene in 40 to 75 seconds. Various quantities of the solution to be tested were added to the system until the maximum velocity was observed. The potency of various solutions of activator could thereby be compared. Since the crude preparation just described was found to be quite reproducible, it was adopted as a temporary standard.

Concentration of Activator—A suspension of 330 gm. of fat-free soy bean meal in 2 liters of water was stirred for 30 minutes, then brought to pH 4.2 with acetic acid, and next heated to boiling for 15 minutes. The supernatant liquid plus the additional solution obtained by squeezing the soy bean curd in cheese-cloth was filtered by suction through paper. Mercuric chloride (4 per cent in H_2O) was added to the liquid until precipitation was complete. The precipitate was centrifuged out. Seven such precipitates were combined and mixed (by shaking with glass beads) with 1 liter of a solution of 1 per cent ammonium acetate and 1 per cent acetic acid in

[3] This reagent consists of 2.5 gm. of ammonium thiocyanate, 5 ml. of concentrated sulfuric acid, 50 mg. of ferrous ammonium sulfate, and enough methyl alcohol to make a volume of 500 ml.

water. The precipitate was recovered by filtration, washed again as before, and then suspended in 200 ml. of the acetate-acetic acid mixture. The suspension was decomposed by H_2S, the mercuric sulfide centrifuged out, and the liquid freed of H_2S by a current of air. Caprylic alcohol reduced foaming. Half its volume of absolute alcohol was added to this liquid, and the precipitate removed by centrifuging. The precipitate was washed with about its own volume of 33 volumes per cent alcohol, the washing being added to the previous supernatant liquid. The combined liquids were then made to 50 volumes per cent of alcohol by the addition of absolute alcohol. The precipitate so produced was washed with twice its volume of 50 per cent alcohol and the washings added to the supernatant liquid obtained in the previous operation. This liquid fraction contained most of the activator. It was precipitated by raising the alcohol concentration to 90 per cent, centrifuged out, washed twice with 90 per cent alcohol, and dried in a vacuum over P_2O_5. The yield was 3.53 gm. of a slightly yellow material.

Character of Activator—Extracts capable of increasing the activity of purified lipoxidase were obtained from many natural sources. Among these were barley malt, rice polishings, several legumes and other vegetables, yeast, rabbit heart muscle, beef muscle, and milk. None of these extracts showed evidence of containing lipoxidase. Efforts to find a known organic substance with this activating effect were unsuccessful. Many of the amino acids, isolated members of the vitamin B complex, uracil, xanthine, adenine, and the common inorganic ions except phosphate were all without action.

Although phosphate buffer by itself has little effect, its presence very markedly reduces the amount of concentrated activator needed for optimum reaction velocity. Citrate also spares activator, but not so markedly as phosphate.

The concentrated material obtained as described from soy bean meal consisted largely of peptides. It contained 16.2 per cent N and 0.068 per cent P. Positive tests were obtained with ninhydrin, Sakaguchi's reagent for arginine, Millon's reagent, biuret reagent (rose color), and nitroprusside (after NaCN). Further evidence of a protein-like nature is shown by inactivation through digestion with papain.

Ultraviolet absorption was observed at 270 to 285 mμ, the tyrosine and tryptophane region, but there was no indication of purine absorption. The Molisch test and the thiochrome reaction were negative. The material contained less than 0.05 per cent of iron and less than 0.01 per cent of manganese. It had no influence on the rate of oxidation of pyrogallol by highly purified horseradish peroxidase and hydrogen peroxide.

The soy bean activator accelerated oxidation of all the oxidizable fats

tested, provided, however, that it was added either to the enzyme or to the fat suspension before the addition of the enzyme. The effect was observed by the rate at which an indicator like carotene or a leucoindophenol was oxidized, and also by measurement of the peroxide content of the fat itself. The enzyme protein had a very slight activity alone, owing possibly to incomplete removal of the activator during purification. When the enzyme was heated with acid, as described in the preparation of crude activator from soy bean meal, the resulting solution had no measurable activating

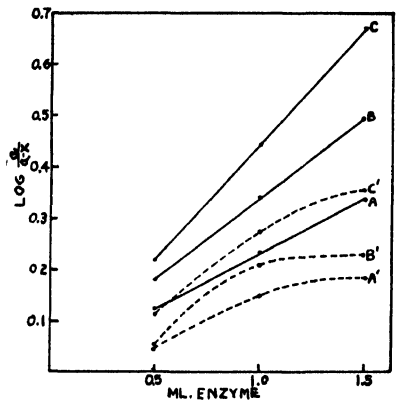


FIG. 2

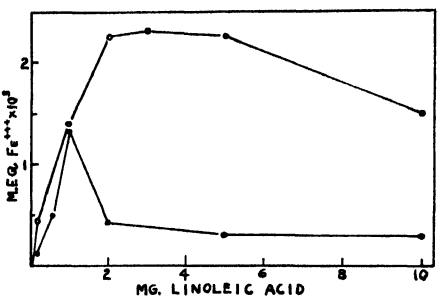


FIG. 3

FIG. 2. The first order nature of the coupled oxidation of carotene in the presence, but not in the absence, of the activator. Curves A, B, and C represent 30, 45, and 60 second reaction times respectively, in the presence of activator. Curves A′, B′, and C′ represent corresponding results in the absence of activator. a = initial carotene concentration, x = carotene destroyed.

FIG. 3. The upper curve represents peroxide formation when various amounts of linoleic acid are used in the presence of the corresponding optimum amounts of activator; the lower curve, peroxide formation when a fixed amount of activator (optimum for 1 mg. of fat) is used with various amounts of fat. Reaction time, 2 minutes. 6 units of lipoxidase were used.

effect, thus indicating that the amount of activator present in purified enzyme is probably small.

The presence of activator appears to change the kinetics of the oxidation of carotene, as shown in Fig. 2. The activated reaction is monomolecular and the velocity constant is directly proportional to the concentration of enzyme. This is not the case when purified enzyme is used without added activator. The complexity of the reaction, however, allows many explanations of these data.

The amount of activator needed to produce maximum peroxide formation

in a given time depends upon the quantity of fat present. If activator and enzyme are both held constant, the maximum peroxide formation occurs in the presence of a definite quantity of fat. Either increase or decrease in the amount of fat results in a decrease in the peroxide formed. The existence of an optimum in the fat concentration, as determined by the rate of carotene destruction, has been reported by other workers (6) who by using a constant quantity of crude enzyme also used a constant quantity of activator along with it. However, when the activator was increased together with the fat, the rate of oxidation increased with substrate concentration until the "zero order" reaction stage was reached (as is usual in enzyme actions), and the "fat concentration optimum" became at least very broad. Crude activator was used in these experiments (Fig. 3).

TABLE II

Activator (Crude Filtrate) Required for Maximum Peroxide Formation with Various Concentrations of Linoleic Acid*

Experiment	Linoleic acid	Enzyme	Activator
	mg.	*units*	*ml.*
A	0.2	6.0	0.2
	1.0	6.0	1.0
	2.0	6.0	1.5
	3.0	6.0	1.9
	5.0	6.0	2.5
	10.0	6.0	4.0
B	1.0	0.75	1.0
	1.0	1.5	0.8
	1.0	3.0	0.8
	1.0	6.0	0.8

* Reaction time, 2 minutes.

The dose of activator may therefore be increased to compensate for an increase in fat. Large doses of activator are apparently inhibitory, giving in effect a broad optimum of activator concentration. The smallest amount of activator required to produce the maximum peroxide formation at a given time may therefore be roughly determined. This relationship is shown in Table II. Furthermore, it is shown that this amount is independent of the amount of enzyme present, within experimental limits.

The "activating" effect of the filtrate from boiled suspensions of soy bean meal is quite different from that of the purified preparation made therefrom. The purified material is about 10 times as effective as the crude (on a dry weight basis) when used in the usual carotene-ethyl linoleate system; that is, in the presence of a cosubstrate and a small amount of fat. If, however, fat is the only substrate present, and the progress of oxidation is measured by

the production of peroxide, the action appears to be very complex, as shown in Fig. 4. Peroxide formation is faster with crude than with purified material (Curves B′ and C′), but in the former case there is evidence of subsequent peroxide destruction. After the first few minutes the total peroxide present remains nearly constant, while with the purified material total peroxide continues to increase for a long time, and eventually reaches much higher values.

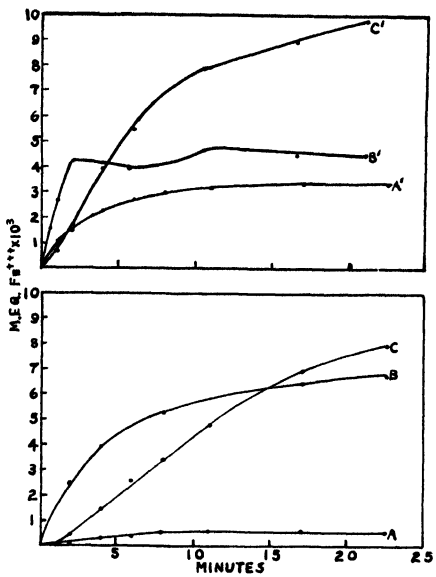


FIG. 4. Comparative effect of optimum amounts of crude and purified activator upon the accumulation of peroxide in the lipoxidase reaction. The upper set of curves was obtained with 15 units of lipoxidase per determination: Curve A′, no activator; Curve B′, 1.5 ml. of crude activator; Curve C′, 0.5 mg. of purified activator. The lower set of curves was obtained with 6 units of lipoxidase per determination: Curve A, no activator; Curve B, 1.5 ml. of crude activator; Curve C, 0.5 mg. of purified activator. 2 mg. of crude linoleic acid were used in each determination.

It therefore seems probable that the "activating" effect of the crude filtrate is due in part to a factor removed during purification. This factor may involve a mechanism whereby the enzyme itself is destroyed when its activity becomes very great (in which case the purified material is merely a "protector"), or it may induce a secondary reaction leading to the rapid disposal of the peroxide formed. It is possible to decide this question, and further study is planned.

Effect of Purothionin on Lipoxidase Action—This protamine, isolated

from wheat flour (13), inhibits oxidation under conditions curiously like those under which the activator accelerates the reaction. For example, the protamine was found to be very inhibitory when about 1 part per million was added to the substrate used for the regular assay. If, however, the protamine was added first to the enzyme plus crude activator, practically no inhibitory effect was observed (Table III). The presence of salts was also necessary for inhibition to take place. Thus, when active enzyme was added to fat and purothionin in water, oxidation occurred. The subsequent addition of phosphate buffer made no difference. But if the salt was added to the system before the enzyme, oxidation was prevented (Table IV).

TABLE III

Inhibition of Carotene Oxidation by Purothionin

	Per cent carotene destroyed in 60 sec.
Enzyme + substrate	68
(Enzyme + 10 γ purothionin) + substrate	66
Enzyme + (substrate + 10 γ purothionin)	5

TABLE IV

Effect of Salt on Inhibition of Carotene Oxidation by Purothionin

Purothionin	Salt	Per cent carotene bleached in 60 sec.
γ		
0	PO_4 buffer (0.5 ml.)	72
10	" " (0.5 ")	4
10	" " (0.5 ") added after enzyme	65
10		73
200		38
200	NaCl (75 mg.)	0
200	NaCN (13 ")	0

A further illustration of the extent to which surface effects appear to be involved in the lipoxidase system is shown by the dependence of purothionin inhibition upon the amount of fat present. Just as more fat requires more activator for its oxidation, so more protamine is required to inhibit that oxidation (Table V). The action of the purothionin is to block the initial fat oxidation, as is shown by determination of the peroxide formed.

Specificity of Lipoxidase—The oxidation of pure fats by the purified enzyme was found to be highly specific. As shown in Table VI, oxidation was confined to acids having more than one double bond, but did not occur with

all of these. Licanic acid was not oxidized, although it contains three. It is noteworthy that the only acids found to be oxidized belong to the group of "essential" fatty acids (16) and that oxidation did occur with each of the three members of this group we were able to test.

TABLE V

Effect of Increased Fat on Inhibition by Purothionin

Purothionin per tube	Per cent carotene bleached in 60 sec.			
	Fat per tube			
	100 γ	200 γ	500 γ	1000 γ
γ				
	78	81	79	84
10	6	17	45	
30	4	2	10	30

TABLE VI

*Oxidation of Unsaturated Fatty Acids and Esters by Soy Bean Lipoxidase**

Substrate	Reaction time	Peroxide formed, m.eq. $Fe^{+++} \times 10^4$
	min.	
Methyl oleate	5	0
Oleic acid	8	0
Erucic "	20	0
Hexadecenoic acid	8	0
Ethyl linoleate†	2	4.2
Linoleic acid (pure)	2	10.3
" " (commercial)	2	8.0
Linolenic " ‡	2	6.5
Methyl arachidonate§	2	15.3
" esters of menhaden oil‖	2	14.4

* 3 units of lipoxidase were used.

† Prepared from tetrabromostearic acid (20).

‡ The initial peroxide value of this quantity of fat was high, approximately 19.9×10^{-4} millicquivalent of Fe^{+++}.

§ From the preparation described by Mowry, Brode, and Brown (21).

‖ Iodine No. 330.

Attempts to find a system in which the fat acts as an intermediary carrier of oxidation have till now been unsuccessful. As an illustration of this, the amount of leuco-*o*-chlorophenol indophenol oxidized was found to vary with the amount of fat (Table VII). After 60 seconds the reaction with lipoxidase was complete or nearly so, and purified peroxidase (devoid of lipoxidase activity) was added. More of the leuco dye was oxidized thereafter,

but the total amount of dye oxidized depended upon the amount of fat present, thus showing no evidence of a cyclic system.

Discussion of Mode of Action of System—The inhibitory effect of fat suspensions in the absence of activator leads to the supposition that fat and enzyme combine, and that such a combination is inactive. This supposition would explain why the activator is not effective when added to the system after the enzyme. The activator may therefore act as a protective agent for the enzyme, perhaps against the fat peroxide (or its more active precursor), or possibly against surface denaturation of the enzyme protein on the fat particles. Evidence that acceleration may be brought about in the latter manner rests on the observation that the presence of activator protects solutions of purified enzyme in water against destruction by shaking. Further evidence is found in Fig. 4. On the other hand, the crude soy bean

TABLE VII

Coupled Oxidation of Leuco-o-chlorophenol Indophenol by Lipoxidase and Peroxidase through Linoleic Acid

Linoleic acid	Dye oxidized*		
	Lipoxidase only, in 60 sec.	Lipoxidase and peroxidase	
		In 120 sec.	At end-point
mg.	γ	γ	γ
100	1 (Reaction complete)	7.5	9
200	2.5 " "	11	20
300	7.5 (" not complete)	34	40

* Each colorimeter tube contained 1.33 mg. of leuco dye, linoleic acid, about 30 units of lipoxidase, and 1.58×10^{-2} unit (22) of purified horseradish peroxidase in a volume of 13 ml. of 0.01 M phosphate, pH 6.5.

filtrate contains some factor that definitely accelerates one or more of the reactions occurring in this system.

It seems unlikely that activator and enzyme form a complex which is stable when joined to the fat, for in that case a quantity of enzyme fully activated for one amount of fat should produce no less effect on a greater amount. It is pertinent to this question, however, to remember that the only fats attacked contain two double bonds with an intervening saturated carbon atom.[4] It is conceivable that the two necessary double bonds must be so spaced that both exist at the fat-water interface. In such a case the old two anchorage theory of von Euler and Josephson (23) might apply; at one double bond the enzyme is attached, at the other, the activator. Since

[4] A previous view (14, 15) that one double bond (the 9–10 bond, *cis* configuration) in a fatty acid is sufficient to define an oxidizable substrate must be abandoned, for oleic acid is not oxidized.

oleic acid and similar substances are definitely inhibitory, this may designate the site of enzyme attachment.

On the other hand, the question of specificity may be linked simply with the oxidizability of the substrate. It is well known, for instance, that linoleic and linolenic acids are far more susceptible to non-enzymatically catalyzed autoxidation than oleic acid (24, 25).

It is probable that the action of lipoxidase is confined to the "essential" fatty acids. If this is the case, it is also probable (but quite unproved) that the rôle of such acids in animal metabolism is one of intermediate catalysis in some form of tissue oxidation.

The work on the specificity of lipoxidase required the use of very pure specimens of fatty acids or their esters. It is a pleasure to thank Dr. W. G. Rose and Mr. C. E. Swift of this Bureau for the specimens of licanic acid and methyl oleate used in this work and for tetrabromostearic acid which was used in the preparation of ethyl linoleate. The other fatty acids and esters used were furnished by Dr. J. B. Brown of The Ohio State University to whom we are very grateful. Our thanks are also due to Mr. M. K. Walden of this Laboratory for his valuable assistance throughout the work.

BIBLIOGRAPHY

1. Hauge, S. M., and Aitkenhead, W., *J. Biol. Chem.*, **93**, 657 (1931).
2. Hauge, S. M., *J. Biol. Chem.*, **108**, 331 (1935).
3. André, E., and Hou, K., *Compt. rend. Acad.*, **194**, 645 (1932); **195**, 172 (1932).
4. Craig, F. N., *J. Biol. Chem.*, **114**, 727 (1936).
5. Sumner, J. B., and Dounce, A. L., *Enzymologia*, **7**, 130 (1939).
6. Sumner, J. B., and Sumner, R. J., *J. Biol. Chem.*, **134**, 531 (1940).
7. Tauber, H., *J. Am. Chem. Soc.*, **62**, 2251 (1940).
8. Strain, H. H., *Carnegie Inst. Washington Yr. Bk.*, **39**, 151 (1940).
9. Sumner, R. J., *J. Biol. Chem.*, **146**, 215 (1942).
10. Süllmann, H., *Helv. chim. acta*, **24**, 1360 (1941).
11. Sumner, R. J., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 14 (1943).
12. Reiser, R., and Fraps, G. S., *J. Assn. Off. Agric. Chem.*, **26**, 186 (1943).
13. Balls, A. K., Hale, W. S., and Harris, T. H., *Cereal Chem.*, **19**, 279 (1942).
14. Strain, H. H., *J. Am. Chem. Soc.*, **63**, 3542 (1941).
15. Sumner, R. J., *J. Biol. Chem.*, **146**, 211 (1942).
16. Burr, G. O., *Federation Proc.*, **1**, 224 (1942).
17. Fraps, G. S., and Kemmerer, A. R., *Ind. and Eng. Chem., News Ed.*, **19**, 846 (1941).
18. Young, C. A., Vogt, R. R., and Nieuwland, J. A., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 198 (1936).
19. Süllmann, H., *Helv. chim. acta*, **25**, 521 (1942).
20. McCutcheon, J. W., Organic syntheses, New York, **22**, 75 (1942).
21. Mowry, D. T., Brode, W. R., and Brown, J. B., *J. Biol. Chem.*, **142**, 671 (1942).
22. Balls, A. K., and Hale, W. S., *J. Assn. Off. Agric. Chem.*, **16**, 445 (1933).
23. von Euler, H., and Josephson, K., *Z. physiol. Chem.*, **133**, 279 (1923); **162**, 85 (1926).
24. Franke, W., *Ann. Chem.*, **498**, 129 (1932).
25. Warburg, O., *Z. physiol. Chem.*, **92**, 231 (1914).

THE PREPARATION OF DEUTERIO CHOLESTEROL*

By KONRAD BLOCH and D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, June 14, 1943)

The study of chemical transformations in the living cell with the aid of the isotope technique requires the preparation of deuterio compounds which contain deuterium atoms linked to carbon atoms. The carbon-deuterium linkages must be stable in the sense that the deuterium atoms will not be replaced by hydrogen atoms on solution in water. In the case of simple organic compounds, standard synthetic procedures will often give the desired compound. The conversion of oleic acid into 9,10-dideuterio stearic acid by the catalytic reduction with deuterium is a simple matter; the preparation of $C_{17}D_{35}COOH$ would obviously be more difficult, as no appropriate hydrogen-free starting substance is available. Its total synthesis from carbon and deuterium, though conceivable, is certainly not practicable. These considerations apply with even greater force to complicated natural products, such as the sterols.

Experiments with heavy hydrogen have revealed a new type of chemical reaction, the exchange reaction, which can be utilized for the preparation of deuterio compounds. The general exchange reaction may be written,

$$AH + DY \rightleftharpoons AD + HY$$

In the water system where Y is either OH or OD, the rate of reaction will depend on the nature of the linkage between A and H. If the hydrogen atom is attached to a paraffinic carbon not adjacent to carbonyl groups, the reaction rate is negligibly small. Cholesterol and fatty acids do not exchange even at elevated temperatures (1), but in the case of fatty acids this reaction may be catalyzed by active platinum (2).

When platinum is used as the catalyst, the organic compound is suspended or dissolved in heavy water and heated at as high a temperature as is consistent with its stability. Deuterium of the heavy water exchanges with the hydrogen of the organic compound. The reaction, of which the detailed mechanism is not known, proceeds well with hydrocarbons (3) and aliphatic acids (2) though not with alcohols (4).

During the study of the metabolism of cholesterol the need arose for deuterio cholesterol. The only obvious approach to its preparation was

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

an exchange reaction with natural cholesterol. Many attempts in this direction were unsuccessful. When suspensions of cholesterol in heavy water were heated in the presence of active platinum, no exchange took place, even at 200°. Increasing the solubility of cholesterol in the medium by addition of alcohol was without effect. In other experiments, selenium was tested as a catalyst for the reaction. At temperatures below the point at which cholesterol is rapidly dehydrogenated by selenium, a slow exchange was observed.

Greater success was encountered with mixtures of water and acetic acid in which the platinum-catalyzed exchange reactions were found to proceed at a rate sufficient to give the desired deuterio cholesterol in practical yields. Degradation of this cholesterol (5) indicates that deuterium has been introduced into both the ring structure and the side chain. No exchanges

TABLE I

Selenium-Catalyzed Exchange Reaction

Experiment No.	Cholesterol	Selenium	Water		Temperature	Time of experiment	Deuterium in cholesterol	
	gm	*gm.*	*ml.*	*atom per cent D*	*°C.*	*hrs*	*atom per cent*	*atom equivalent**
1	1.8	0.2	1.0	98	110	72	0.22	0.1
2	1.0	0.2	0.3	99	230	4	1.7	0.8
3†	5.0	5.0	100	4	220	2	0.05	0.6
4‡	2.0	0.5	25	4	125	96	0.00	0.0

* Atom equivalents $= \frac{\text{per cent D in cholesterol}}{\text{per cent D in water}} \times 46.$

† 10 ml. of ethyl alcohol were added to increase the solubility of cholesterol.

‡ 25 ml. of ethyl alcohol were added to increase the solubility of cholesterol.

occurred with cholesteryl chloride and *i*-cholesteryl methyl ether in heavy water in the presence of active platinum.

EXPERIMENTAL

Selenium Catalysis—Several exchange reactions were carried out in the presence of selenium, which is known to dehydrogenate cholesterol at 320°. It was thought possible that at lower temperatures the hydrogen atoms of the cholesterol would be sufficiently activated to permit exchange with the water. The results of these experiments are shown in Table I. In the eighth column are given the deuterium concentrations of the isolated cholesterol samples. In the last column is calculated the number of hydrogen atoms of the cholesterol which have exchanged on the assumption that the partition coefficient of the deuterium between cholesterol and water is 1. In Experiments 3 and 4 alcohol was added to increase the

solubility of cholesterol in the medium. In Experiments 2 and 3 there was a small exchange, but the reaction was obviously very slow. In Experiment 2 the volume of water employed was so small that at 230° it was all present as vapor.

The experimental data of the exchange reaction in acetic acid-water mixtures are given in Table II. The procedure employed in the isolation of cholesterol from such reaction mixtures will be illustrated by one representative experiment (No. 5, Table II). 1.25 gm. of platinum oxide suspended in a mixture of 40 ml. of deuterio acetic acid (containing 60 atom

TABLE II

Platinum-Catalyzed Exchange Reaction of Cholesterol with Deuterium Oxide in Acetic Acid

Experiment No.	Acetic acid concentration*	Temperature	Time	Platinum per gm. cholesterol	D in water	Deuterium in cholesterol		Sterols pptd. from reaction mixture as digitonides	Cholesterol isolated
		°C.	*days*	*gm.*	*atom per cent*	*atom per cent*	*atom equivalents*†	*per cent*	*per cent*
1	100	127	2	0.15	17‡	0.38	1.0	68	37
2	70	127	2	0.15	30	0.86	1.3	58	25
3	70	127	2	0.15	30	0.88	1.3	60	22
4	50	127	2	0.25	30	1.80	2.8	28	5
5	70	127	3	0.10	100	4.16	1.9		45
6	70	127	4	0.30	88	5.70	3.0		12
7	70	127	6	0.15	6.75	0.29	2.0	28	15
8	70	127	6	0.30	6.75	0.38	2.6	20	12
9	70	100	6	0.15	6.75	0.07	0.5	70	50

* Except in Experiments 1 and 5, normal acetic acid was used for the exchange reaction.

† Atom equivalents $= \frac{\text{per cent D in cholesterol}}{\text{per cent D in water}} \times 46.$

‡ Atom per cent D in carbon-bound hydrogen of deuterio acetic acid.

per cent D) and 13 ml. of 99 per cent D_2O were reduced with ordinary hydrogen; the excess hydrogen was replaced by nitrogen and 12.5 gm. of cholesterol were added to the mixture. The reaction flask was evacuated, sealed, and shaken for 3 days in an oven at 127°. The solvent was distilled off *in vacuo*; the residue, which contained appreciable amounts of cholesteryl acetate, was taken up in ether, filtered, evaporated, and treated with 400 ml. of 95 per cent ethanol containing 8 gm. of potassium hydroxide for 4 days at room temperature. The alcoholic solution was chilled, the crystals were filtered off, and a second crop was obtained by concentrating the filtrate. A third crop was secured from the mother liquor by dilution

with water and extraction with ether. The combined crude sterol fractions (7.5 gm.), after several recrystallizations from acetone, yielded 4.9 gm. of cholesterol, m.p. 148° corrected, $[\alpha]_D^{26} = -39°$ (2 per cent in chloroform).

From the combined mother liquors more sterol was precipitated by digitonin, regenerated, and purified via the dibromide. The resulting 0.6 gm. of cholesterol, m.p. 147.5° corrected, brought the total yield to 5.5 gm. or *44 per cent.* The deuterium concentration was 4.16 atom per cent excess. The effects of changes in temperature, time, acetic acid concentration, and amount of active catalyst are given in Table II. In Experiments 4 and 6 acetate was isolated as silver deuterio acetate, the samples of which contained 6.9 and 9.9 atom per cent deuterium, respectively.

To ascertain the relative distribution of deuterium in the molecule, the deuterio cholesterol of Experiment 5 was converted to cholesteryl chloride and subjected to thermal degradation as described previously (5). The hydrogen in the resulting mixture of isooctane and isooctene showed a higher level of deuterium than that of the hydrocarbon $C_{19}H_{30}$, although more isotope was actually present in an equimolecular quantity of the latter.

C_8H_{18}. 4.93% D, 18 × 0.0493 = 0.887 atom D
$C_{19}H_{30}$. 3.66% " 30 × 0.0366 = 1.098 atoms "

The deuterium content of a compound made up of the fragments of C_8H_{18} and $C_{19}H_{30}$, as calculated from their isotope content, would be 4.14 per cent. This theoretical value agrees closely with that observed for the deuterio cholesterol.

DISCUSSION

The resistance of cholesterol to exchange reactions cannot be due to the inhibiting action of the alcohol group, since cholesteryl chloride and *i*-cholesteryl methyl ether, neither of which contains a hydroxyl group, do not undergo the exchange reaction.[1] Nor is it a general property of all steroids.[2] Mutual solubility of reactants is not necessary for an exchange reaction. For example, Horrex and Polanyi have shown that the water-cyclohexane exchange is readily catalyzed by platinum (3). However, the addition of acetic acid to the water-cholesterol-platinum system accelerates the exchange reaction. Simultaneously it leads to a marked destruction of the cholesterol molecule. Comparison of Experiments 1, 2, 3, and 4

[1] Unpublished experiments.

[2] When 0.9 gm. of desoxycholic acid, 6 cc. of water containing 6.75 atom per cent excess D, and 0.5 gm. of active platinum were shaken for 5 days at 112°, there was introduced into the acid 0.43 atom per cent excess deuterium.

illustrates the effect of varying acetic acid concentration. As the concentration of acetic acid is lowered, the number of deuterium atoms introduced rises from 1 in 100 per cent acetic acid to 1.3 in 70 per cent acetic acid, to 2.8 in 50 per cent acetic acid. At the same time the yield of digitonin-precipitable sterols, as well as of cholesterol itself, drops sharply as the acetic acid concentration is lowered. The decomposition proceeds so far that in 50 per cent acetic acid-water mixture only 5 per cent of the cholesterol could be recovered. It appears from all the experiments that the rate of introduction of deuterium into the cholesterol is roughly parallel to the extent of decomposition. This rate can be affected by change of temperature (see Experiments 7 and 9), acetic acid concentration (see Experiments 1, 2, 3, and 4), and amount of platinum (see Experiments 7 and 8). Possibly the two processes have some common steps; activation of the cholesterol molecule sufficient to allow exchange must be of such a nature as to make it possible for irreversible chemical reactions to occur.[3]

SUMMARY

1. Treatment of cholesterol with heavy water and selenium leads to a slow introduction of deuterium into the molecule.

2. Platinum is unable to catalyze the exchange between cholesterol and water alone, but the addition of acetic acid to the water-cholesterol-platinum system results in an exchange reaction.

3. Deuterium is introduced by this reaction into both the side chain and the ring system of cholesterol.

4. Simultaneously with the exchange a destruction of cholesterol occurs. The destruction seems to run parallel to the amount of deuterium introduced into the remaining cholesterol.

BIBLIOGRAPHY

1. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121**, 235 (1937).
2. van Heyningen, W. E., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **125**, 495 (1938).
3. Horrex, C., and Polanyi, M., *Mem. and Proc. Manchester Lit. and Phil. Soc.*, **80**, 33 (1935–36).
4. Stetten, D., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 347 (1940)
5. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **145**, 625 (1942).

[3] The chemical nature of the products formed during the treatment of cholesterol in acetic acid solutions with active platinum is under investigation.

THE BIOLOGICAL CONVERSION OF CHOLESTEROL TO CHOLIC ACID*

By KONRAD BLOCH, BENJAMIN N. BERG, AND D. RITTENBERG

(From the Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, June 14, 1943)

It has recently been demonstrated (1) that acetate is utilized for the *in vivo* synthesis of cholesterol, but the mechanism by which steroids are formed by animal tissues is unknown. No evidence is available as to whether bile acids and steroid hormones arise independently by a similar condensation of small molecules or whether they are products of cholesterol metabolism.

Of the three types of cyclopentenophenanthrene derivatives, cholesterol is the most abundant in the animal organism and, in contrast to bile acids and hormones, is found in all tissues and body fluids. Cholesterol is continuously being synthesized from small molecules and the total amount of sterol in the animal body appears to remain rather constant under normal dietary conditions. Data on the rate of bile acid production in the intact animal are not available, as accurate determinations of the relative proportions which are reabsorbed or excreted in the feces are not feasible. The balance type of experiments could, therefore, hardly be expected to provide an answer as to the biological relationship between sterols and bile acids. Attempts to correlate cholesterol supply and bile acid production in animals with fistulas have been unsuccessful (2, 3) and experiments of this type have never been carried out with intact animals.

It was thought that cholesterol labeled with deuterium should be of aid in throwing light on the relationship between cholesterol and bile acids; appearance of deuterio cholic acid after its administration would constitute clear evidence of direct conversion. This substance, prepared by the method described in the foregoing article (4), was administered to a dog previously cholecystonephrostomized by the procedure of Kapsinow (5). Since sterol absorption after oral administration was uncertain under the experimental conditions, the deuterio cholesterol was given by intravenous infusion.

Cholic acid isolated from urine collected during the experimental period was analyzed for deuterium. After careful purification, the absence of deuterio cholesterol as a contaminant was demonstrated by the "washing

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

out" procedure (6), with ordinary cholesterol. It is evident from the data in Table I that bile acid had been formed from cholesterol.

3 days after the last administration of deuterio cholesterol, the dog was exsanguinated and cholesterol was isolated from the blood and various

TABLE I

Atom Per Cent Excess Deuterium in Cholic Acid and Bile Cholesterol after Intravenous Injection of Deuterio Cholesterol (4.16 Atom Per Cent Excess Deuterium)

Sample No.	Days after 1st injection	Cholic acid	Bile cholesterol
I	1, 2	0.17	0.13
II	4	0.24	0.50
III	5, 6	0.16	0.29

TABLE II

Atom Per Cent Excess Deuterium in Tissue Cholesterol after Intravenous Injection of Deuterio Cholesterol (4.16 Atom Per Cent Excess Deuterium)

Organ	D_2 excess	Organ	D_2 excess
Red blood cells	0.31	Pancreas	0.25
Plasma	0.33	Adrenals	0.30
Liver	0.71	Omentum	0.20
Kidney	0.31	Testis	0.15
Lungs	2.00	Brain	0.00
Heart	0.39	Spinal cord	0.00
Spleen	0.46		

TABLE III

Atom Per Cent Excess Deuterium in Fecal Sterols Excreted after Intravenous Injection of Deuterio Cholesterol

Days after 1st injection	Excess D_2 in fecal sterol
1	0.00
2	0.04
4	0.13
6	0.17

organs. The isotope concentrations found in these samples of cholesterol are given in Table II.

The feces were collected during the experimental period. Total sterols were isolated from 24 hour specimens. The isotope concentrations found in these samples are given in Table III.

EXPERIMENTAL

In a male adult dog weighing 10 kilos an anastomosis of the gallbladder to the pelvis of the right kidney was established and the common duct doubly ligated and divided. The dog was kept on a diet of raw beef. 2 weeks following the operation, the animal received daily by intravenous infusion an emulsion of 1 gm. of deuterio cholesterol containing 4.16 per cent D_2 for 3 consecutive days. The ring system of this cholesterol contained 3.66 atom per cent excess D and the side chain 4.93 atom per cent excess D (4). 1 gm. of sterol and 1 gm. of lecithin were suspended in 100 ml. of 0.05 M phosphate buffer, pH 7.3, and emulsified with the aid of a Waring blendor. Homogeneous, filtrable emulsions were thus obtained. 100 ml. of emulsion were administered by infusion into the femoral vein in the course of 30 minutes. The dog was placed in a metabolism cage and kept for 3 days following the last injection, when it was killed. Urine and feces were collected during the experimental period.

Isolation of Cholic Acid—Urines of 48 hour periods were pooled, acidified with dilute sulfuric acid, and extracted continuously with ether for 24 hours. The ether extracts were brought to dryness and the residue was saponified by being heated in 20 per cent aqueous NaOH solution for 24 hours on the steam bath. After exhaustive extraction with ether to remove unsaponifiable material, the alkaline solution was acidified and the mixture of bile acids and fatty acids was taken up in ether. The ether was distilled off, the residue was dissolved in dilute ammonia, and saturated barium hydroxide solution added until no more precipitation occurred. The barium salts of desoxycholic acid and fatty acids were removed by filtration, and the cholic acid was extracted with ether from the acidified filtrate. The ethereal solution was dried and concentrated, when cholic acid crystallized out. In order to eliminate any possible contamination with deuterio cholesterol, the cholic acid was redissolved in ether and twice the weight of ordinary non-isotopic cholesterol was added. The ether solution was then extracted with dilute sodium carbonate and the alkaline solution acidified. The precipitated cholic acid was taken up in ether, and the ether solution was dried and brought to a small volume. Cholic acid crystallized from the solution.

Of the three cholic acid samples isolated from the dog urine, Samples I and III were treated in this manner, whereas Sample II was not "washed out."

The analytical data for the cholic acid samples were:

Sample I—C 70.5, H 9.5, m.p. 198°
Sample II—M.p. 190°
Sample III—C 71.0, H 9.6, m.p. 199°
Calculated. C 70.6, H 9.8

The deuterium concentrations found in the cholic acids are listed in Table I.

Isolation of Cholesterol from Urine—The ether extracts, obtained from the alkaline hydrolysate of the initial urine extracts were brought to dryness, taken up in alcohol, and precipitated with digitonin. Sterol Samples I, II, and III were extracted from the same runs as cholic acid Samples I, II, III. Total amounts isolated from urine were, Sample I, 37 mg. of sterol; Sample II, 15 mg. of sterol; Sample III, 42 mg. of sterol. These samples are referred to as bile cholesterol and their deuterium concentrations are given in Table I.

Isolation of Fecal Sterols—The feces collected during a 24 hour period were dehydrated by two acetone extractions and then extracted with ether continuously for 24 hours. The ether and acetone extracts were saponified with 10 per cent alcoholic KOH and an aliquot of the unsaponifiable material precipitated with digitonin. For deuterium analysis the sterol digitonides were burnt. The isotope concentrations of the sterols were calculated from that of the sterol digitonides and are given in Table III.

Cholesterol from Tissue Constituents—The tissues were saponified with 10 per cent potassium hydroxide in ethanol and cholesterol obtained from the unsaponifiable fraction in the usual fashion. Cholesterol was isolated either as the digitonide or, when sufficient material was available, recrystallized as the free compound until the melting point reached 147°. The isotope analyses of the cholesterol samples are given in Table II.

DISCUSSION

The concentrations of deuterium present in all three samples of isolated bile acid are significant and of the same order of magnitude as those of blood and "bile" cholesterol.[1] Specifically, the cholic acid excreted during the last 48 hours before the killing of the animal contained one-half as much deuterium as the cholesterol excreted simultaneously and also one-half as much as the blood cholesterol immediately before the death of the dog. In contrast, the liver sterol contained 4 times as much isotope as this particular sample of cholic acid.

A conversion of cholesterol to cholic acid would necessitate shortening of the sterol side chain by 3 carbon atoms, introduction of hydroxyl groups at carbon atoms 7 and 12, saturation of the $\Delta^{5,6}$ double bond, and epimerization of the hydroxyl group at carbon atom 3.

Assuming that in the administered deuterio cholesterol all the hydrogen atoms of the isooctyl side chain have the same average isotope concentration, *i.e.* 4.93 per cent, and that all carbon-bound hydrogen in the nucleus

[1] Since the quantities of urinary sterol excreted by normal animals are insignificant, the sterol appearing in the urine under our experimental conditions may be assumed to be of biliary origin. It is therefore referred to as bile cholesterol.

contained an average of 3.66 per cent deuterium (4), it will be possible to calculate the isotope concentration of cholic acid if formed from cholesterol. This value will obviously depend on the chemical reactions taking place in the transformation, but in any case the cholic acid will of necessity contain less deuterium than the cholesterol from which it is formed. A minimum of 5, and possibly 6, normal hydrogen atoms will be introduced into the nucleus in the course of the following reactions. (1) Oxygen atoms, with normal hydrogen, are introduced at positions 7, 12, and 24 respectively, *i.e.* 3 normal H atoms. (2) Saturation of the $\Delta^{5,6}$ double bond in cholesterol involves uptake of 2 normal hydrogen atoms. (3) If the epimerization of the hydroxyl group at carbon atom 3 were to involve the intermediary formation of a 3-keto compound, at least 1 isotopic hydrogen atom would be replaced by a normal one. On the other hand, epimerization may be the result of a Walden inversion which could occur without loss of deuterium. A cholic acid formed as the result of these reactions could retain 8 of the hydrogen atoms (with 4.93 atom per cent D) originally present in the cholesterol side chain and 27 hydrogen atoms of the original nucleus containing 3.66 atom per cent D_2, while at least 5 hydrogen atoms were derived from the normal hydrogen of the organism. Its deuterium content will then be at most 83 per cent of that of the cholesterol from which it was formed. Thus the cholic acid excreted during the last 48 hours of the experiment could not have contained more than 0.24 per cent D if derived from the circulating cholesterol (*i.e.* that of plasma, red cells, and bile) and not more than 0.59 per cent D if the liver cholesterol had served as the immediate precursor. The cholic acid actually contained 0.16 per cent D, *i.e.* respectively about two-thirds and one-quarter of these values. These values[2] were arrived at by correcting only for those isotopic hydrogen atoms which must have been lost by reactions (1) and (2) (above). Additional loss of deuterium could be visualized if the introduction of hydroxyl groups at carbon atoms 7 and 12 involved either desaturation and subsequent hydrogenation or intermediary formation of 7,12-keto compounds as precursors of cholic acid.

Cholesterol had evidently been deposited in the liver, since at the end of the experiment, 3 days after the last administration of deuterio cholesterol, the liver sterol still contained more than twice as much isotope as the circulating cholesterol. Thus the replacement of cholesterol present in the liver by newly formed cholesterol proceeds at a slow rate, indicating a low metabolic activity of the sterol deposited in the liver following the intravenous administration of deuterio compound. It cannot be decided

[2] Dilution of the bile acids newly formed by those originally present must have been insignificant, since bile had been excreted by the dog for 2 weeks before deuterio cholesterol was administered and ligation of the bile duct prevented reabsorption of bile acids.

whether the deposition of relatively inert liver cholesterol was due to the fact that cholesterol and not cholesterol ester was administered. The finding that the red blood cell cholesterol (which consists of free cholesterol only) and the plasma cholesterol (two-thirds of which is esterified) contain identical isotope concentrations seems to exclude this possibility.

The cholesterol excreted with the bile, having the same isotope value as that of the blood and only about 40 per cent of that of liver cholesterol, can hardly be of hepatic origin. The function of the liver with regard to bile cholesterol seems to be excretory only.

Similarly, the bile acids, though formed in the liver, could arise from the circulating cholesterol as the immediate precursor rather than from that stored in the liver. Our data on the relative isotope concentrations in circulating cholesterol and cholic acid do not belie such a concept, although they supply no proof for it.

The deuterium concentrations found in the cholesterol of various tissues of the animals represent the isotope content 3 days after the last administration of deuterio cholesterol. As can be seen from Table III, there is wide variation in the ability of different tissues to remove cholesterol from the circulating blood. The relative storage capacity is most pronounced in the lungs and liver. All other tissues, with the exception of brain and spinal cord, had incorporated cholesterol from the circulation, although to varying degrees. The absence of knowledge relating to the metabolic function of cholesterol precludes an interpretation of these data. However, the complete absence of deuterio cholesterol in brain and spinal cord is notable. It illustrates the lack or paucity of metabolic interchange between the sterol of the central nervous system and that of the blood. Slow rates of metabolism in brain have been observed previously by Waelsch, Sperry, and Stoyanoff (7), who demonstrated that cholesterol is synthesized very slowly, if at all, in the brain of adult rats. Cholesterol of the central nervous system seems to be the most inert of all tissue constituents which have as yet been studied; it is not regenerated and does not interchange with the dietary cholesterol at appreciable rates.

From the data in Table III it is evident that isotopic sterol had been secreted into the lumen of the intestine, although the experimental conditions prevented secretion of bile into the intestine. These findings confirm those of Sperry (8) who observed excretion of sterols in the feces of a dog with bile fistula kept on a sterol-free diet.

SUMMARY

1. Cholesterol containing 4.2 per cent deuterium was administered intravenously to a dog in which an anastomosis between the gallbladder and the pelvis of the kidney had been established.

2. Cholic acid isolated from the dog urine had an isotope concentration of the same order of magnitude as the blood or bile cholesterol, demonstrating the biological conversion of sterol to bile acid. Assuming that the circulating sterol provided the immediate precursor, it was calculated that a minimum of two-thirds of the cholic acid arose by degradation of cholesterol.

3. The distribution of the administered cholesterol was determined in various organs. The highest concentration was found in the lung, followed by the liver. All other organs, with the exception of the central nervous system, contain approximately the same concentrations as that of the blood. No deuterio cholesterol had been deposited in brain or spinal cord.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **143**, 297 (1942); **145**, 625 (1942).
2. Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, **38**, 421 (1919).
3. Enderlen, E. S., Thannhauser, S J., and Jenke, M., *Arch. exp Path. u. Pharmakol.*, **130**, 308 (1928).
4. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **149**, 505 (1943).
5. Kapsinow, R., Engle, L. P., and Harvey, S. C., *Surg., Gynec. and Obst.*, **39**, 62 (1924).
6. Schoenheimer, R., and Rittenberg, D , *J. Biol. Chem.*, **120**, 155 (1937).
7. Waelsch, H., Sperry, W. M., and Stoyanoff, V. A., *J. Biol Chem.*, **135**, 291 (1940).
8. Sperry, W. M., *J. Biol. Chem.*, **71**, 351 (1926–27).

THE UTILIZATION OF THE METHYL GROUPS OF CHOLINE IN THE BIOLOGICAL SYNTHESIS OF METHIONINE*

By SOFIA SIMMONDS, MILDRED COHN, JOSEPH P. CHANDLER, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, June 14, 1943)

The demonstration that homocystine plus choline could replace methionine in the diet of the growing rat led to the suggestion (1) that the methyl groups of choline are utilized in the biomethylation of homocysteine to methionine. Direct proof of this postulated transfer of methyl groups is offered in the present communication. The occurrence in the animal body of the reverse transmethylation, namely the utilization of the methyl group of methionine in the formation of choline, has already been established (2) by experiments in which isotopic choline was found in the tissues subsequent to the administration of isotopic methionine.

The method employed in the present study depended on tracing methyl groups labeled with deuterium from the choline in the diet to the methionine in the tissue proteins. Since a procedure for the isolation of methionine itself from small amounts of proteins in sufficiently high yields was not attained, the methyl group cleaved from the methionine in the tissue proteins was isolated by an adaptation of the Baernstein procedure (3, 4) for the quantitative determination of methionine in proteins. By this method the methionine is converted by hydriodic acid to methyl iodide and homocysteine thiolactone. In the present study, the methyl iodide formed during the acid digestion of the isolated tissue proteins was passed into an alcoholic solution of trimethylamine and the tetramethylammonium iodide so obtained was either analyzed directly for deuterium or was converted to tetramethylammonium chloride chloroplatinate prior to the deuterium analysis.

When synthetic deuteriomethionine was subjected to this treatment in control experiments the carbon-deuterium bonds in the S-methyl group were sufficiently labilized to allow some loss of deuterium. The values we obtained for the isotopic content of tissue methionine are therefore minimal values. Tetramethylammonium iodide prepared from deuteriomethionine retained 66 to 72 per cent of the calculated amount of deuterium. The

* The authors wish to acknowledge grants-in-aid by The Nutrition Foundation, Inc., and Eli Lilly and Company which have contributed to the support of this investigation.

retention of all the deuterium in the quaternary salt prepared from a synthetic sample of deuteriomethyl iodide indicated that deuterium-hydrogen exchange occurs during the treatment with strong acid. The labilization of the carbon-deuterium bond concomitant with the *in vitro* cleavage of methionine was striking in view of the fact that the *in vivo* cleavage involves no such labilization. It should also be pointed out that the carbon-deuterium bond in the N-methyl groups of deuteriocholine is not labilized when deuteriocholine is subjected to a variety of vigorous conditions such as boiling with 1 N hydrochloric acid, boiling with 5 per cent barium hydroxide, or treatment with hot alkaline permanganate solution (2). It has been reported that deuterium-hydrogen exchange occurs in dimethyl sulfone and sodium methylsulfonate in alkaline solutions (5). A deu-

TABLE I

Feeding Experiments with Deuteriocholine and Homocystine*

Rat No.	Duration of experiment	Change in body weight	Deuteriocholine ingested	Body choline		Body creatine		Body methionine	
				Deuterium in chloroplatinate	Deuterium in methyl groups	Deuterium in creatinine K picrate	Deuterium in methyl group	Deuterium in tetramethyl-ammonium salt	Deuterium in methyl of methionine
	days	*gm.*	*mg. per day*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*
500	23	90–100	50	33.3 ± 0.4	51.8 ± 0.6	5.0 ± 0.1	20.0 ± 0.4	4.2 ± 0.2	16.8 ± 0.8
505	56†	82–111	25	42.0 ± 0.6	65.3 ± 0.9	6.1 ± 0.2	24.4 ± 0.8	4.4 ± 0.4	17.6 ± 1.6

* The deuterium content of the methyl groups of the choline fed was 84.5 atom per cent.

† From the 1st to 6th day, Rat 505 received 50 mg. of deuteriocholine per day; from the 7th to 12th day, 50 mg. of ordinary choline per day; from the 13th to 56th day, 25 mg. of deuteriocholine per day.

terium-hydrogen exchange was also observed by us when deuteriomethionine was heated in 20 per cent sodium hydroxide.

EXPERIMENTAL

Feeding of Deuteriocholine and Homocystine—Two young rats, weighing about 85 gm., were placed on the homocystine-containing diet, described in a previous paper (2), supplemented with deuteriocholine. Deuteriocholine was synthesized from deuteriomethyl iodide and aminoethanol (2). The amount of isotopic choline fed, the duration of the experiment, and the change in body weight of the two animals are given in Table I. At the close of the experimental period the animals were sacrificed and choline and creatine were isolated from the tissues. The deuterium contents of

the body creatine and of the urinary creatinine have been reported in a preceding paper (2) but the creatine values are given again for comparison. Tissue proteins were subjected to treatment with hydriodic acid as described below to convert the methyl group of methionine to the tetramethylammonium salt. In the case of Rat 500 the tetramethylammonium iodide itself was analyzed for deuterium, while the iodide obtained from Rat 505 was converted to tetramethylammonium chloride chloroplatinate prior to deuterium analysis. The deuterium contents of the isolated compounds are given in Table I.

TABLE II

Feeding Experiments with Deuteriocholine in Absence of Dietary Homocystine for 3 Day Period*

Group	S-Amino acid supplement	Rat No.	Change in body weight	Total deuteriocholine ingested	Body choline		Body creatine		Body methionine	
					Deuterium in chloroplatinate	Deuterium in methyl groups	Deuterium in creatinine K picrate	Deuterium in methyl group	Deuterium in tetramethylammonium salt	Deuterium in methyl of methionine
			gm.	*gm.*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*
A	1.4% methionine	749	50–53	0.075	9.3 ±0.1	14.5 ±0.2	0.17 ±0.08	0.68 ±0.32	0.34 ±0.05	1.36 ±0.20
		750	50–53	0.075	9.5 ±0.1	14.8 ±0.2	0.24 ±0.05	0.96 ±0.20		
B	None	751	52–52	0.075	10.6 ±0.1	16.5 ±0.2	0.38 ±0.05	1.52 ±0.20	0.15 ±0.06	0.60 ±0.24
		752	50–52	0.069	9.2 ±0.2	14.4 ±0.2	0.26 ±0.05	1.04 ±0.20		

* The deuterium content of the methyl groups of the choline fed was 84.5 atom per cent.

Feeding of Deuteriocholine in Absence of Homocystine—Four young rats, weighing about 50 gm., were placed on the sulfur-amino acid-free basal diet (6) to which were added 25 mg. of deuteriocholine per day. The rats were divided into two groups and the diet of each group was supplemented as follows: Group A, 1.4 per cent methionine and Group B, none. At the end of 3 days all the animals were sacrificed and tissue choline and creatine were isolated. The tissue proteins from the two rats in a given pair were pooled in order to provide sufficient material for the methionine procedure. The deuterium contents of all the isolated compounds are given in Table II.

Isolation of Choline from Body of Rat—Choline was isolated as choline chloroplatinate from a boiling alcohol extract of the ground tissues by the

method described previously (2). In Tables I and II, the deuterium contents of the isolated compounds are listed.

$C_{10}H_{28}O_2N_2PtCl_6$.	Rat 500.	Calculated,[1]	N 4.48;	found, N 4.30
"	" 505.	"	" 4.46; "	" 4.43
"	" 749.	"	Pt 31.50;	found, Pt 31.47
"	" 750.	"	" 31.50; "	" 31.20
"	" 751.	"	" 31.50; "	" 31.80
"	" 752.	"	" 31.50; "	" 32.00

Isolation of Creatine from Tissues—Creatine was isolated by the method of Foster, Schoenheimer, and Rittenberg (7) as the double salt, creatinine potassium picrate, from the same alcohol extract used for the isolation of choline. The purity of the creatinine potassium picrates was determined by the Jaffe color reaction. The deuterium contents of the isolated creatinine salts are given in Tables I and II.

TABLE III

Control Experiments on Stability of Carbon-Deuterium Bond in S-Methyl Group of Methionine

Deuterium in S-methyl group of initial methionine (*A*)	Deuterium in tetramethylammonium salt	Deuterium in S-methyl group of final methionine (*B*)	$B/A \times 100$
atom per cent	*atom per cent*	*atom per cent*	
3.84 ± 0.05	0.69 ± 0.03	2.76 ± 0.12	71.9
41.6 ± 0.6	6.9 ± 0.2	27.6 ± 0.8	66.3
4.56 ± 0.06	0.78 ± 0.12	3.12 ± 0.48	68.4

Control Experiments on Deuterium-Hydrogen Exchange—Three samples of deuteriomethionine were digested with hydriodic acid and the methyl iodide samples obtained were converted to the chloroplatinate of tetramethylammonium chloride. The deuterium contents of these salts, given in Table III, ranged from 66 to 72 per cent of the calculated values. The loss of deuterium did not occur during the conversion of tetramethylammonium iodide to the chloride chloroplatinate, since chloroplatinates prepared from samples of deuteriotetramethylammonium iodide of known deuterium content always contained the theoretical amount of the isotope. Nor does the preparation of tetramethylammonium iodide by addition of methyl iodide to an alcoholic solution of trimethylamine involve labilization of the carbon-deuterium bond, for a sample of the quaternary iodide prepared from deuteriomethyl iodide of known deuterium content also contained the theoretical amount of deuterium.

[1] All calculated values are based on the increased molecular weight due to deuterium in the molecule.

A sample of deuteriomethionine was dissolved in 20 per cent sodium hydroxide and the solution boiled under a reflux for 23 hours. The deuterium content of the methionine isolated from this digest was 57.5 per cent of that of the initial material.

Isolation of S-Methyl Group of Methionine from Tissue Proteins—The residue from the hot alcohol extraction of the rat tissue was extracted three times with cold 6 per cent trichloroacetic acid over a 3 day period to remove non-protein nitrogen. The trichloroacetic acid-insoluble material was then reextracted with hot alcohol and dried. The tissue proteins so obtained represented about 14.5 per cent of the body weight of the smaller (50 gm.) rats and about 16.5 per cent of the body weight of the larger (100 gm.) rats.

About 7 gm. of rat proteins were then digested in 25 cc. of boiling 57 per cent hydriodic acid for 6 to 7 hours and the methyl iodide formed was collected in alcoholic trimethylamine. The apparatus used for the hydriodic acid treatment was a modification of that described by Baernstein (4). The digestion flask was connected in the usual way to an absorption train consisting of four scrubbers. The construction and contents of the first two scrubbers were identical with those used in the ordinary Baernstein procedure. The third and fourth scrubbers consisted of widened test-tubes similar to those used in the determination of methylimide by the method of Pregl and Lieb (8). A stop-cock was inserted between the second and third scrubbers so that the third and fourth scrubbers could be separated from the rest of the apparatus. These two scrubbers contained a 5 per cent alcoholic solution of trimethylamine. Because both methyl iodide and trimethylamine as well as ethyl alcohol are easily volatilized, the third and fourth scrubbers were cooled in solid carbon dioxide during the digestion period. At the resulting low temperature a large part of the carbon dioxide gas used to sweep the volatile products of hydrolysis out of the digestion flask was absorbed along with the methyl iodide by the alcoholic trimethylamine solution. At the close of the digestion period the trimethylamine scrubbers were removed from the train and allowed to stand in their solid carbon dioxide baths for about 30 hours. During this time the solid carbon dioxide slowly evaporated and, as the contents of the scrubbers warmed to room temperature, the dissolved carbon dioxide gradually escaped from the alcoholic solution.

About 100 mg. of crude tetramethylammonium iodide were obtained after evaporation of the alcoholic trimethylamine solution and several washings of the residue with cold absolute alcohol. Trial runs on methionine alone and on rat tissues indicated that the crude tetramethylammonium iodide obtained represented about 80 per cent of the methionine calculated on the basis of the homocysteine thiolactone in the hydriodic

acid digest. The quaternary ammonium iodide could be purified by recrystallization but it proved inconvenient for use in the deuterium analysis because of its high iodine content. Therefore, the crude iodide was washed free of iodine with cold absolute alcohol and was converted to the chloride by treatment with silver chloride. From an alcoholic solution of the chloride the chloroplatinate was prepared and this salt was analyzed for deuterium. The deuterium contents of the various samples obtained from tissues are given in Tables I and II.

$C_4H_{12}NI$.	Rat 500.	Calculated,[1] I 63.1; found, I 63.6
$C_8H_{24}N_2PtCl_6$.	" 505.	" Pt 35.01; " Pt 35.33
"	Rats 749, 750.	Calculated,[1] Pt 35.07; found, Pt 34.79
"	" 751, 752.	" " 35.07; " " 34.77

DISCUSSION

The utilization of the methyl groups of dietary choline in the biosynthesis of methionine has now been established by the demonstration that the tissue proteins of rats fed deuteriocholine and homocystine contain deuteriomethionine. The *in vivo* synthesis of methionine proceeds regardless of the absence of homocystine in the diet, since a small amount of deuteriomethionine was found in the tissue proteins when deuteriocholine was fed on a sulfur-amino acid-free basal diet. Furthermore, even when adequate methionine is added to the deuteriocholine diet methyl transfer from choline to methionine continues.[2]

It becomes obvious from these results that the process of transmethylation from dietary choline to methionine is not induced by the presence of homocystine in the diet. That is, the presence in the diet of a methyl acceptor is not requisite for the phenomenon of transmethylation to methionine to make its appearance. Apparently, a precursor of methionine, undoubtedly homocysteine, is formed by the animal during the catabolism of methionine, thus enabling methionine to be resynthesized with the methyl group supplied by choline. Continual synthesis of methionine occurs although more than enough methionine is supplied in the diet—a fact which places transmethylation from choline to methionine in the class of "automatic and non-interruptable biochemical processes" (9).

In all of these experiments, the transmethylation to creatine as well as to methionine has been followed. As previously reported (2), the methyl group from dietary choline appears in the creatine of the tissues and the creatinine of the urine when deuteriocholine was fed with homocystine. As shown in Tables I and II, transmethylation from choline to creatine

[2] Experiments in which deuteriomethionine and ordinary choline were fed together show that the formation of choline from methionine proceeds even with an adequate dietary supply of choline.

also occurs, though to a lesser extent, when homocystine is excluded from the basal diet or when ordinary methionine is in the diet instead of homocystine.

SUMMARY

Direct proof of the transfer of methyl groups from choline to methionine in the rat is presented by the demonstration of the presence of the deuteriomethyl group in tissue methionine subsequent to the ingestion of deuteriocholine and homocystine. Furthermore, this transmethylation reaction was shown to occur when deuteriocholine was fed without homocystine in the diet and even when ordinary methionine was fed with deuteriocholine.

The authors wish to thank Dr. J. R. Rachele of this laboratory for some of the microanalyses.

BIBLIOGRAPHY

1. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
2. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).
3. Baernstein, H. D., *J. Biol. Chem.*, **106**, 451 (1934).
4. Baernstein, H. D., *J. Biol. Chem.*, **115**, 25 (1936).
5. Hochberg, J., and Bonhoeffer, K. F., *Z. physik. Chem., Abt. A*, **184**, 419 (1939).
6. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135**, 223 (1940).
7. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
8. Roth, H., Quantitative organic microanalysis of Fritz Pregl, translated by Daw, E. B., Philadelphia, 3rd English edition, Fig. 52 (1937).
9. Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 415 (1940).

NOTE ON THE USE OF THE *o*-PHENANTHROLINE FERROUS COMPLEX AS AN INDICATOR IN THE CERIC SULFATE TITRATION OF BLOOD SUGAR

By DOUGLAS A. MacFADYEN and DONALD D. VAN SLYKE

(From the Alfred I. du Pont Institute of the Nemours Foundation, Wilmington, Delaware, and the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, June 24, 1943)

Miller and Van Slyke (1) have published a direct microtitration method for blood sugar in which the ferrocyanide formed by reduction of ferricyanide is titrated with ceric sulfate. As indicator setopaline C[3] was recommended after trial of numerous other oxidation-reduction indicators. The constitution of this indicator appears to be a trade secret, and the indicator is now off the market. However, it has been found that *o*-phenanthroline,[1] synthesized by Blau (2) and studied with regard to its oxidation properties by Walden, Hammett, and Chapman (3), serves even better than the setopaline C[3].

The indicator is used in the form of a ferrous complex, which is prepared in 0.025 M solution as follows: 6.95 gm. of $FeSO_4 \cdot 7H_2O$ are dissolved and made up to 1 liter with water. Into the solution are stirred 14.85 gm. of *o*-phenanthroline monohydrate, $C_{12}H_8N_2 \cdot H_2O$, until all is dissolved.

The color change at the end-point in the sugar titration is sharp, from golden brown to light yellow. On standing the golden brown slowly returns. The end-point, however, is stable for at least a minute.

BIBLIOGRAPHY

1. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114**, 583 (1936).
2. Blau, F., *Monatsh. Chem.*, **19**, 647 (1898).
3. Walden, G. H., Hammett, L. P., and Chapman, R. P., *J. Am. Chem. Soc.*, **53**, 3908 (1931).

[1] The indicator, either in solid form or prepared in 0.025 M ferrous complex solution, as described above, can be obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio.

FURTHER STUDIES ON THE RÔLE OF POTASSIUM AND OTHER IONS IN THE PHOSPHORYLATION OF THE ADENYLIC SYSTEM*

By PAUL D. BOYER, HENRY A. LARDY, AND PAUL H. PHILLIPS

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, June 9, 1943)

Recently it was demonstrated that potassium had an essential rôle in the transfer of phosphate from 2-phosphopyruvate or 3-phosphoglycerate to the adenylic system (1). Earlier publications have indicated that other substances might replace potassium in some of its physiological effects. Ohlmeyer and Ochoa (2) have shown that glucose phosphorylation by yeast was stimulated interchangeably by manganese, magnesium, potassium, and ammonium ions. Conway *et al.* (3) and Fenn and Haege (4) have found that ammonium ions will replace potassium in yeast and muscle cells. Hence experiments were undertaken to study the relationships of Mg^{++}, Mn^{++}, and NH_4^+ to the effect of K^+ on phosphorylation.

Several studies have shown that oxidation of pyruvate may be coupled with phosphorylations (5–7). In view of the demonstrated importance of K^+ for anaerobic phosphorylation, studies were made to ascertain the relationship of K^+ to the phosphorylation accompanying pyruvate oxidation.

Since adenosinetriphosphatase has been found to be inhibited by K^+ (8), it was felt desirable to present evidence which shows that the effect of K^+ on phosphorylation was not the result of an inhibition of adenosinetriphosphatase.

In the course of these experiments it was noted that adenosine triphosphate additions were markedly superior to adenylic acid additions for the catalysis of the phosphate transfer from 2-phosphopyruvate to creatine. Thus this difference in the effect of adenosine triphosphate and adenylic acid was further studied.

In addition to the results of the previously mentioned experiments further data concerning the stimulating effect of K^+ and the inhibiting effect of Ca^{++} and other inhibitors on phosphate transfer are presented in this paper.

Methods and Materials

The preparation of tissues and phosphorus esters and the determination of phosphocreatine were made as described previously (1). The adenylic

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

acid used was prepared by the method of Kerr (9). The sodium pyruvate used as a substrate was prepared from pyruvic acid by the method of Robertson (10).

In experiments in which phosphorus fractionation studies are reported, relatively small amounts of organic phosphorus compounds were added with the tissue preparations. Thus nearly all the phosphorus esters present were derived from the added 3-phosphoglycerate. The phosphoglycerate which disappears can be accounted for almost entirely as 2-phosphopyruvate, adenosine triphosphate, phosphocreatine, and inorganic phosphate. Hence conventional fractionation and hydrolysis procedures for the estimation of these phosphorus esters can be applied with good results.

Phosphoglycerate was estimated as the differences in the total P and the P hydrolyzable in 180 minutes at 100° in N HCl. Disappearance of phosphoglycerate was measured by the increase in the P hydrolyzable in 180 minutes. For the determination of adenosine triphosphate and of inorganic P the calcium precipitate from the phosphocreatine determination was washed once with a mixture of 1 part of the precipitating agent and 4 parts of water, and the inorganic and 7 minute-hydrolyzable phosphorus determined on the precipitate. 2-Phosphopyruvate was estimated as the difference in the amount of P hydrolyzable in 180 minutes and the amount present as phosphocreatine, inorganic phosphate, and adenosine triphosphate. In a number of cases this value was checked by the alkaline iodine hydrolysis procedure of Lohmann and Meyerhof (11).

The experiments were performed with either dilute, homogenized, fresh rat muscle or with enzyme preparations from acetone precipitates of muscle extracts. The acetone precipitates were prepared from rat muscle which was coarsely chopped, and washed once with distilled water. The tissue was then finely ground in 5 volumes of calcium-free Ringer-phosphate solution at pH 7, the mixture centrifuged, and the precipitate discarded. 4 volumes of cold acetone were added to the supernatant solution and the precipitate was collected by centrifuging, washed once with acetone, and stored at 0°. The dialyzed extract from the acetone precipitate was prepared by dialysis for 48 hours, against frequently changed distilled water, in the refrigerator.

All experiments were conducted at 38°. The experiments on anaerobic transfers were done in test-tubes with shaking in a constant temperature bath. The dilute homogenized tissue preparations and the enzyme preparations from acetone precipitates of muscle extracts showed no oxygen uptake under the conditions of the experiments; hence exclusion of air was unnecessary. Oxidative experiments were conducted manometrically by use of a Warburg apparatus in the conventional manner.

Results

Specific Action of Potassium—The transfer of phosphate from 3-phosphoglycerate to creatine has been shown to take place by the following series of reactions (11–15).

(1) 3-Phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate $\rightleftharpoons$ 2-phosphopyruvate

(2) 2-Phosphopyruvate + adenosine diphosphate $\rightleftharpoons$ pyruvate + adenosine triphosphate

(3) Adenosine triphosphate + creatine $\rightleftharpoons$ adenosine diphosphate + phosphocreatine

Dephosphorylation of the adenosine triphosphate may also occur by the action of adenosinetriphosphatase if this enzyme is present, in which case inorganic phosphate is liberated.

The equilibrium in Reaction 1 is such that only small amounts of 2-phosphoglycerate are present (16). Adenosine triphosphate is required only in catalytic amounts for the over-all reaction (Nos. 1 to 3). Thus in the study of this system the measurement of the phosphorus present as phosphocreatine, phosphoglycerate, 2-phosphopyruvate, and inorganic phosphate gives an indication of the mode of action of any added activator or inhibitor of the phosphate transfers involved.

The results of experiments on the relationship of K^+, Mg^{++}, Mn^{++}, and NH_4^+ to the phosphate transfer from 3-phosphoglycerate to creatine are given in Table I. The levels of Mn^{++}, Mg^{++}, and NH_4^+ used were comparable to those used by Ohlmeyer and Ochoa in their experiments with yeast (2).

The results with fresh tissue show that addition of K^+ markedly accelerated the disappearance of phosphoglyceric acid. This probably resulted because K^+ accelerated the phosphorylation of the adenylic system. That this might be the case is indicated by previous results (1) showing that K^+ was necessary for the phosphate transfer from 2-phosphopyruvate to creatine but not for the transfer from adenosine triphosphate to creatine. The marked increase in inorganic phosphate which appeared in the presence of K^+ is likewise most readily explained by an increased transfer of phosphate to the adenylic system and its subsequent liberation by the active adenosinetriphosphatase which the fresh tissue preparation contained. This increase in inorganic phosphate was opposite to the effect that would have resulted if K^+ accelerated creatine phosphorylation by inhibiting adenosine triphosphate breakdown by adenosinetriphosphatase.

The higher 2-phosphopyruvate concentration in the absence of K^+ (Table I) showed that the rate of 2-phosphopyruvate formation did not limit the rate of phosphocreatine formation in the absence of K^+. Ex-

periments with dialyzed preparations from acetone precipitate of muscle extracts, in confirmation of results of Utter and Werkman (17) obtained with preparations from *Escherichia coli*, have shown that K^+ has no effect on the rate of attainment of the equilibrium between 3-phosphoglycerate and 2-phosphopyruvate.

K^+ also had a marked stimulatory effect on the phosphate transfer from 3-phosphoglycerate to creatine when dialyzed extracts of acetone precipi-

TABLE I

Effects of Potassium on Phosphorylation in Relation to Magnesium, Manganese, and Ammonium Ions

Each tube contained 60 micromoles of creatine, 0.2 micromole of adenosine triphosphate, and 0.005 M sodium phosphate buffer. In Experiment 1 the enzyme source was homogenized, fresh muscle tissue, and in Experiment 2 dialyzed extract of an acetone precipitate. 20.5 and 10.3 micromoles of 3-phosphoglycerate were added to each tube in Experiments 1 and 2 respectively. The total volume was 1.0 cc., incubation time 20 minutes.

Enzyme source	KCl added	$MgCl_2$ added	Other additions	3-Phosphoglycerate disappeared	Phosphocreatine formed	"Phosphopyruvate" present	Inorganic phosphate appeared
	M	*M*		*micromoles*	*micromoles*	*micromoles*	*micromoles*
Fresh tissue	0	0.005		4.6	0.6	2.3	1.7
	0	0.005	1×10^{-4} M Mn^{++}	4.2	0.3	2.4	1.5
	0	0.005	4×10^{-4} M Mn^{++}	3.9	0.3	2.0	1.6
	0	0.005	0.075 M NH_4^+	10.5	3.6	2.2	4.5
	0.05	0.005		10.1	3.4	1.2	5.6
	0.5	0.005		5.6	2.0	2.0	1.5
Dialyzed extract	0.05	0		1.0	0.2	0.2	0.8
	0	0.005		3.5	1.2	1.9	0.3
	0.05	0.005		4.9	4.2	0.5	0.6
	0	0.01		3.2	1.2	1.6	0.4
	0.05	0.01		4.7	3.3	1.0	0.3
	0	0.005	2×10^{-4} M Mn^{++}	1.9	1.0	0.7	0.2
	0.05	0.005	2×10^{-4} M Mn^{++}	3.7	3.3	0.2	0.2
	0	0.005	0.15 M NH_4^+	3.6	3.0	0.3	0.3

tates were used, as shown in Table I. Since these preparations had no adenosinetriphosphatase activity, this stimulating effect of K^+ could not be due to an inhibition of adenosinetriphosphatase. In the samples without K^+ additions, some phosphate transfer took place. Thus the principal effect of K^+ was to accelerate the rate of transfer. With non-dialyzed extracts from acetone precipitates only slight activation by K^+ could be demonstrated.

The results show that manganese in levels of 0.1 to 0.4 micromole per cc.

would not replace K^+ in the experiments with either fresh tissue or dialyzed extracts of acetone precipitates, and did not accelerate the phosphate transfer in the presence of K^+ and Mg^{++}. Increased levels of Mg^{++} would not replace K^+ or cause an increase in the phosphate transfer in the presence of K^+. The low rate of phosphate transfer by the dialyzed extracts in the presence of K^+ but absence of Mg^{++} indicated that the dialyzable metallic ions were fairly completely removed. In other experiments it was shown that Mn^{++} will replace Mg^{++} in this system of phosphate transfers. Thus these results show that K^+ was essential in addition to either Mg^{++} or Mn^{++} for the rapid phosphorylation of the adenylic system.

In contrast to the results with Mg^{++} and Mn^{++}, the data in Table I show that NH_4^+ would replace K^+ entirely both with fresh tissue preparations and with dialyzed extracts from acetone precipitates.

The inhibiting effect of K^+ on the activity of adenosinetriphosphatase, reported by Mehl and Sexton (8), has been confirmed by us. However, the inhibition was not marked until concentrations of 0.2 M or greater were reached. Concentrations of K^+ well below 0.2 M will markedly stimulate phosphorylation. For this reason, and those given above, it may be concluded that the stimulating effect of K^+ on phosphate transfer from 2-phosphopyruvate to creatine was not due to inhibition of adenosine triphosphate breakdown. It is of interest that K^+ in high concentrations also inhibits the transfer of phosphate from 2-phosphopyruvate to creatine.

Comparative Catalytic Effect of Adenosine Triphosphate and Adenylic Acid—In experiments with dialyzed extracts of acetone precipitates prepared from muscle it was found that adenylic acid would not replace adenosine triphosphate in the transfer of phosphate from 2-phosphopyruvate to creatine. The results given in Table II show the striking contrast found when additions of adenylic acid and adenosine triphosphate were compared. Thus it follows that in this system adenosine diphosphate accepted phosphorus from 2-phosphopyruvate much more rapidly than would adenylic acid.

Action of Inhibitors—Calcium has been shown to inhibit the stimulating effect of K^+ on phosphorylation (1). As Ca^{++} has been found to activate adenosinetriphosphatase (18), studies were made to see if the inhibition by Ca^{++} might be due to an increased breakdown of adenosine triphosphate into adenylic acid and inorganic phosphate.

The breakdown of added adenosine triphosphate by homogenized fresh rat muscle tissue under conditions similar to those of the phosphorylation experiments was not accelerated by additions of Ca^{++} to a concentration of 0.004 M. Evidently the tissue preparations contained sufficient Ca^{++} for maximum activation of the adenosinetriphosphatase.

Experiments were also made to determine the effect of Ca^{++} on the

transfer of phosphate from adenosine triphosphate to creatine both with and without the adenosine triphosphate concentration as the limiting factor in the transfer. The results of these experiments are given in

TABLE II

Comparative Ability of Adenylic Acid and of Adenosine Triphosphate to Catalyze Phosphate Transfer from 2-Phosphopyruvate to Creatine

Each tube contained 15 micromoles of creatine, 0.005 M sodium phosphate buffer, pH 7.4, 0.005 M Mg^{++}, and 10 micromoles of 3-phosphoglycerate in a total volume of 0.5 cc. The extracts of acetone precipitates used as enzyme sources were dialyzed for 24 hours and 96 hours in Experiments 1 and 2 respectively.

Experiment No	KCl added	Adenosine triphosphate added	Adenylic acid added	Phosphocreatine formed		
				10 min.	20 min.	30 min.
	M	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*micromoles*
1	0	0.33			1.51	
	0.05	0.33			3.72	
	0		0.33		0.05	
	0.05		0.33		0.20	
2	0	0.16		0.42		1.19
	0.05	0.16		1.03		2.51
	0		0.16	0.08		0.17
	0.05		0.16	0.15		0.56

TABLE III

Lack of Effect of Calcium on Transfer of Phosphate from Adenosine Triphosphate to Creatine

Each tube contained 30 micromoles of creatine, 0.005 M Mg^{++}, and 0.5 mg. of homogenized, fresh muscle tissue in 0.06 M Na_2HPO_4, pH 8.7. The total volume was 0.5 cc.

Adenosine triphosphate added	$CaCl_2$ added	Phosphocreatine formed	
		5 min.	10 min.
micromoles easily hydrolyzable P	*M*	*micromoles*	*micromoles*
3.0		0.52	0.88
1.5		0.48	0.71
3.0	0.004	0.55	0.84
1.5	0.004	0.48	0.65

Table III. The data show that Ca^{++} did not effect the transfer of phosphate from adenosine triphosphate to creatine. When the level of added easily hydrolyzable P as adenosine triphosphate was decreased from 3 to 1.5 micromoles, a decreased formation of phosphocreatine resulted, but Ca^{++} had no effect on the rate of transfer. If Ca^{++} accelerated the break-

down of adenosine triphosphate, a decreased formation of phosphocreatine would be expected in the presence of Ca^{++} when the concentration of adenosine triphosphate limited the rate of transfer.

Further evidence that the inhibitory effect of Ca^{++} was the result of some mechanism other than activation of adenosine triphosphate breakdown

TABLE IV

Effect of Various Inhibitors on Transfer of Phosphate from 3-Phosphoglycerate to Creatine

Each tube contained 60 micromoles of creatine, 0.2 micromole of adenosine triphosphate, 0.005 M Mg^{++}, 0.005 to 0.1 M phosphate buffer, pH 7.4, and 0.05 M KCl. Either 12.5 mg. of homogenized fresh muscle tissue or a dialyzed extract from an acetone precipitate was used as an enzyme source. The total volume was 1.0 cc., incubation time 20 minutes.

Experiment No.	Enzyme source	Inhibitor added	3-Phosphoglycerate added	3-Phosphoglycerate disappeared	Phosphocreatine formed	"Phosphopyruvate" present
		M	*micromoles*	*micromoles*	*micromoles*	*micromoles*
1	Fresh tissue	0	10	7.3	3.2	
	" "	0.004 $CaCl_2$	10	3.6	0.3	
2	Dialyzed extract	0	20.7	10.3	10.4	2.9
	" "	0.004 $CaCl_2$	20.7	8.5	4.6	3.8
	" "	0.008 " *	20.7	6.7	3.0	3.8
	" "	0 012 " *	20.7	5.2	1.9	3.7
3	Fresh tissue	0	20.5	9.5	3 2	0 9
	" "	0.008 oxalate	20.5	3.9	0.3	2.9
4	Dialyzed extract	0	10 3	5.0	4.1	0.5
	" "	0.004 oxalate	10.3	3.0	1.2	1.4
	" "	No KCl added	10.3	3.4	1.2	2.0
5	" "	0	20.7	9.4	6.5	3.4
	" "	0.1 M NaCl	20 7	8.1	5.1	3.6
	" "	0.1 " KCl	20.7	9.2	5.9	3.4

* With a $CaCl_2$ concentration of 0.008 or 0.012 M a precipitate of calcium phosphate formed.

was that Ca^{++} had an inhibitory effect when extracts of acetone precipitates, which had negligible adenosinetriphosphatase activity, were used.

Additional studies were made to ascertain whether the effect of Ca^{++} was the result of inhibition of the formation of 2-phosphopyruvate from 3-phosphoglycerate or inhibition of the transfer of phosphate from 2-phosphopyruvate to the adenylic system. The results of phosphorus fractionation studies with various levels of Ca^{++} present and with an acetone precipitate of muscle extract as an enzyme source are shown in Table IV. The calcium markedly inhibited the formation of phospho-

creatine and the disappearance of phosphoglycerate was lessened. In all cases relatively high amounts of 2-phosphopyruvate were present (estimated both by indirect calculation and alkaline iodine hydrolysis; the results by the two procedures agreed within about 10 per cent). Thus the inhibiting action of Ca^{++} was exerted on the transfer of phosphate from 2-phosphopyruvate to creatine. The results given above showed that Ca^{++} did not effect the transfer from adenosine triphosphate to creatine. Therefore, Ca^{++} exerted its inhibitory effect on the transfer of phosphate from 2-phosphopyruvate to the adenylic system. The inhibitory action of Ca^{++} was thus directly opposed to the action of K^+; both ions acted upon the same transfer.

During the course of experiments on oxidation and phosphorylation, oxalate was added to ascertain whether a possible stimulation of phosphorylation might result from Ca^{++} removal by oxalate. No stimulation, but a greater inhibition traceable to the oxalate added, was noted. Hence, the effect of oxalate on the anaerobic phosphorylation was studied. The results shown in Table IV indicated that oxalate did not act by slowing the formation of 2-phosphopyruvate. The effects of adding oxalate were found similar to those produced by not adding K^+ (Experiment 4, Table IV). Other studies on oxalate have shown that it does not accelerate adenosine triphosphate breakdown. Lohmann and Meyerhof (11) have previously shown that oxalate inhibits dephosphorylation of 2-phosphopyruvate.

Concentrations of K^+ greater than the optimum concentrations or additions of Na^+ inhibit the phosphate transfers. The results given in Table IV showed that increased K^+ concentrations or Na^+ additions did not lessen the amount of 2-phosphopyruvate present.

The effects of added Na^+, K^+, and $PO_4^{\equiv}$, and of K^+ in the presence of NaCl and Na_2HPO_4, on the phosphate transfer from 3-phosphoglycerate to creatine by homogenized fresh tissue are shown in Fig. 1. The molarities of the added substances were in addition to the 0.005 M potassium phosphate buffer, pH 7.4, already present. Under these conditions optimum phosphorylation was obtained with a K^+ concentration as low as 0.05 M. In the presence of 0.06 M Na_2HPO_4, pH 8.7, the optimum K^+ concentration was 0.2 M, although much smaller levels of K^+ markedly increased phosphate transfer.

The results show that addition of relatively high concentrations of Na^+ caused a marked inhibition of phosphocreatine formation. With 0.1 M NaCl present in the medium, stimulation by K^+ still occurred, but the rate of phosphorylation obtainable was far below that obtained in the absence of added Na^+. This was similar to the effects of K^+ when Ca^{++} was present as an inhibitor (1). Thus the effects of these inhibitors cannot be

overcome by K^+ additions, although the amount of phosphate transfer can be increased by K^+ additions if the K^+ concentration is below optimum.

If the KCl was replaced by equimolar concentrations of potassium phosphate buffer, pH 7.4, the inhibition of the phosphate transfer by inorganic phosphate can be demonstrated (Fig. 1). Smaller concentrations of phosphate were stimulatory to the transfer. The inhibitory effect of Na^+ and of $PO_4^{\equiv}$ were similar to results obtained by Ohlmeyer and Ochoa in the study of the transfer of phosphate from 2-phosphopyruvate to glucose by yeast (2).

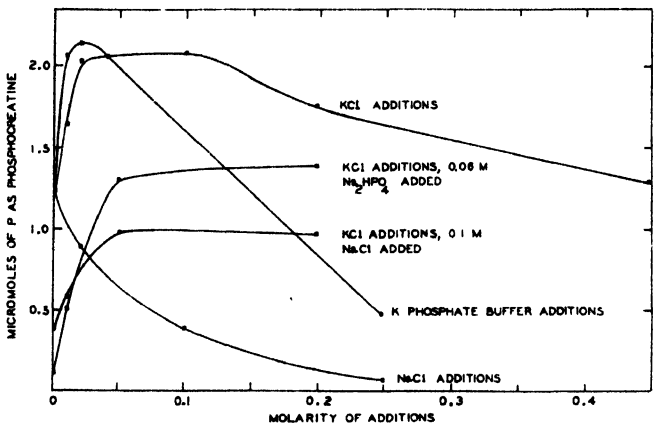


FIG. 1. The effect of potassium, sodium, and inorganic phosphate on the transfer of phosphate from 3-phosphoglycerate to creatine. Each tube contained 60 micromoles of creatine, 0.005 M Mg^{++}, 0.2 micromole of adenosine triphosphate, 17.0 micromoles of 3-phosphoglycerate, and 12.5 mg. of homogenized fresh rat muscle tissue. The total volume was 1.0 cc., incubation time 20 minutes. The molarity of added substances was in addition to the 0.004 M potassium phosphate buffer, pH 7.4, and other substances already present. The concentration of Na^+ added with the 3-phosphoglycerate and other additions was calculated to be about 0.1 M.

Relation of Potassium to Oxidative Phosphorylation—Studies on pyruvate oxidation were made with minced muscle preparations. With homogenized preparations the oxidation of pyruvate was slow unless high tissue concentrations were used. No coupling of phosphorylation with oxidation by homogenized fresh tissues could be demonstrated, but with minced preparations a phosphorylation coupled with pyruvate oxidation could be obtained.

In the experiments on pyruvate oxidation malate was added to catalyze the oxidation (19, 6). Additions of cocarboxylase and cozymase[1] were tried in several experiments, but they did not stimulate the rate of oxidation by minced tissue.

[1] Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

The results of several experiments on pyruvate oxidation are given in Table V. In the absence of K^+ very little phosphocreatine was formed, while in the presence of K^+ phosphorylation of added creatine occurred. The rate of oxidation was not markedly affected by K^+, but K^+ was necessary for phosphorylation to accompany oxidation. In the absence of pyruvate some endogenous phosphorylation occurred in the presence but not in the absence of K^+.

TABLE V

Relation of Potassium to Phosphorylations Accompanying Pyruvate Oxidation

Each Warburg flask contained 120 micromoles of creatine, 0.2 micromole of adenosine triphosphate, 0.003 M Mg^{++}, 0.033 M phosphate buffer, pH 7.4, and 75 mg. of minced fresh rat muscle. Pyruvate and malate were added from a side arm after equilibration to make concentrations of 0.05 M and 0.002 M respectively. The phosphocreatine formed includes that formed during the preliminary and equilibration period and the 60 minute period during which oxygen uptake was measured.

Experiment No.	KCl added (final molarity)	Other conditions	Phosphocreatine formed	Oxygen consumed
			micromoles	*micromoles*
1	0		0.9	6.1
	0.2		7.1	4.6
	0.2	0.004 M Na oxalate	2.9	4.7
	0.2	No pyruvate added	3.0	2.2
2	0		0.6	4.3
	0.2		8.4	4.0
	0.2		8.2	3.8
	0.2	No pyruvate added	4.1	1.6
3	0.2		8.0	3.9
	0.2	0.008 M $CaCl_2$	0.1	0
	0.2	No pyruvate added	1.8	2.0

The effect of K^+ on the oxidative phosphorylation was inhibited by oxalate, but the oxalate did not decrease the rate of oxidation. Addition of Ca^{++} in amounts which would inhibit phosphorylation also inhibited the O_2 uptake. Adenosinetriphosphatase activity has been found to be unaffected by oxalate, indicating that in the oxidative as well as the anaerobic phosphorylation the effect of K^+ was not the result of adenosinetriphosphatase inhibition.

DISCUSSION

The results of these studies indicate that the effect of K^+ on the phosphorylation of the adenylic system in muscle is a specific one. Although NH_4^+ will replace K^+ in *in vitro* experiments, it is not found in the cell in sufficient concentration to be of any importance in the phosphorylation of the adenylic system. Mg^{++} or Mn^{++} is also essential for the rapid

transfer of phosphate from 2-phosphopyruvate to the adenylic system, as shown by these and earlier studies (11, 13). However, the action of Mg^{++} and Mn^{++} is separate from that of K^+, which is essential in addition to Mg^{++} and Mn^{++}.

The results of our experiments indicate that adenosine diphosphate will accept phosphate from 2-phosphopyruvate much more readily than will adenylic acid, since for the rapid phosphorylation of creatine by dialyzed extracts traces of the higher phosphorylated stages must be added. These results may explain the observation of Cori and coworkers (20) that adenosine triphosphate but not adenylic acid would function in the alternate dephosphorylation mechanism of 2-phosphopyruvate described by Pillai (21) and Meyerhof and Junowicz-Kocholaty (22).

The inhibition of phosphate transfer by Ca^{++} appears to be directly antagonistic to the action of K^+; *i.e.*, it inhibits the transfer of phosphate from 2-phosphopyruvate to the adenylic system. Utter and Werkman found that with preparations from *Escherichia coli* Ca^{++} inhibited the rate of formation of 2-phosphopyruvate from 3-phosphoglycerate (17). It is possible that in our experiments Ca^{++}, in addition to its prominent effect on the transfer of phosphate from 2-phosphopyruvate, may slow the rate of formation of 2-phosphopyruvate. However, this would not be evident or limiting, because the 2-phosphopyruvate is only slowly removed in the presence of Ca^{++}.

It is not probable that the increase in inorganic phosphate noted in experiments with fresh tissue when K^+ was added was the result of the breakdown of adenosine triphosphate by the formation of 1,3-diphosphoglyceric acid which spontaneously decomposes (22). Likewise the small increase in inorganic phosphate in the experiments with acetone precipitates indicates that this dephosphorylation mechanism was nonoperative. These results are as would be expected, since it was found that the dephosphorylation of adenosine triphosphate by this mechanism did not occur unless nearly all the adenylic acid was present as adenosine triphosphate (22). In our experiments the presence of creatine, and in some instances of adenosine triphosphatase, makes this improbable.

The effect of K^+ on the phosphorylation accompanying pyruvate oxidation further emphasizes the essential nature of K^+ in the phosphorylation of the adenylic system. The lack of accumulation of phosphocreatine in the absence of K^+ when pyruvate is oxidized, although oxidation continues unimpeded, is of interest. This indicates that in the absence of K^+ the oxidation may not be coupled with phosphorylation or that a phosphorylated intermediate may accumulate or decompose spontaneously or enzymatically. In the presence of K^+ the adenylic system may be able to accept the phosphate from a phosphorylated intermediate.

Phosphocreatine accumulation in the system we used does not always

accompany pyruvate oxidation by rapidly respiring tissue. In some preparations, even under what we assumed to be favorable conditions, oxidation would continue without phosphorylation resulting. Other investigators have also found that oxidative phosphorylation may be somewhat erratic (19, 23). Further investigations on the mechanism of the coupling of phosphorylation with oxidation of various substrates are necessary.

The rate of 2-phosphopyruvate breakdown by dialyzed acetone precipitates of muscle provides the basis for a quantitative estimation of adenosine-5-phosphoric acid and its homologues in the method published by Schlenk and Schlenk (24). In view of the results of the experiments reported here it would seem advisable that in such an assay the K^+ level should be controlled and it should be recognized that the addition of Na^+, and especially Ca^{++}, with the material to be assayed would deleteriously affect the results.

SUMMARY

Experiments have been conducted with enzyme preparations from muscle to determine the specificity of the accelerating effect of K^+ on the phosphorylation of the adenylic system, to compare the ability of adenylic acid and adenosine triphosphate additions to catalyze phosphate transfer from 2-phosphopyruvate to creatine, to study the effect of K^+ on aerobic as well as anaerobic phosphorylation, and to study the action of Ca^{++} and other inhibitors of the stimulating effect of potassium. The results of these experiments warrant the following conclusions.

1. K^+ markedly accelerates the transfer of phosphate from 3-phosphoglycerate to creatine by either homogenized fresh muscle or by dialyzed extracts of acetone precipitates prepared from fresh muscle. The optimum K^+ concentration under favorable conditions is as low as 0.05 M.

2. The action of K^+ is the result of an accelerating effect on the transfer of phosphate from 2-phosphopyruvate to the adenylic system. K^+ is essential in addition to either Mg^{++} or Mn^{++} for this transfer. However, NH_4^+ will replace K^+ in *in vitro* experiments.

3. Adenosine triphosphate additions are much more effective than adenylic acid additions for catalysis of the transfer of phosphate from 2-phosphopyruvate to creatine.

4. K^+ is necessary for the phosphorylation of creatine to accompany pyruvate oxidation by minced tissue. In the absence of K^+ phosphorylation of creatine does not result, although oxidation is unimpeded.

5. Ca^{++} is directly antagonistic to the effect of K^+. The inhibitory effect of Ca^{++} is due principally to an inhibition of the transfer of phosphate from 2-phosphopyruvate to the adenylic system.

6. The actions of K^+ and of Ca^{++} on phosphorylation in the system studied were not the result of respective inhibition and stimulation of adenosinetriphosphatase. However, K^+ in relatively high concentrations does inhibit adenosinetriphosphatase.

7. Oxalate inhibits the effect of potassium on the phosphate transfer from 2-phosphopyruvate to creatine. It also inhibits the effect of K^+ on the phosphorylations accompanying pyruvate oxidation without affecting the oxygen uptake.

8. Both inorganic phosphate and Na^+ in relatively high concentrations are inhibitory to the transfer of phosphate from 3-phosphoglycerate to creatine.

BIBLIOGRAPHY

1. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 673 (1942).
2. Ohlmeyer, P., and Ochoa, S., *Biochem. Z.*, **293**, 338 (1937).
3. Conway, E. J., O'Brien, M. F., and Boyle, P. J., *Nature*, **148**, 662 (1941).
4. Fenn, W. O., and Haege, L. F., *Federation Proc.*, **1**, pt. 2, 24 (1942).
5. Lipmann, F., *Nature*, **143**, 281 (1939).
6. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, **137**, 343 (1941).
7. Ochoa, S., *J. Biol. Chem.*, **138**, 751 (1942).
8. Mehl, J. W., and Sexton, E. L., *Proc. Soc. Exp. Biol. and Med.*, **52**, 38 (1943).
9. Kerr, S E., *J. Biol. Chem.*, **139**, 131 (1941).
10. Robertson, W. v. B., *Science*, **96**, 93 (1942).
11. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
12. Meyerhof, O., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **4**, 207 (1935).
13. Lehmann, H., *Biochem. Z.*, **281**, 271 (1935).
14. Lohmann, K., *Biochem. Z.*, **233**, 460 (1931).
15. Parnas, J. K., Ostern, P., and Mann, T., *Biochem. Z.*, **275**, 74 (1934).
16. Meyerhof, O., and Schulz, W., *Biochem. Z.*, **297**, 60 (1938).
17. Utter, M. F., and Werkman, C. H., *J. Biol. Chem.*, **146**, 289 (1942).
18. Bailey, K., *Biochem. J.*, **36**, 121 (1942).
19. Belitzer, V. A., *Bull. biol. et méd. exp. URSS*, **7**, 11 (1939); *Chem. Abst.*, **33**, 6417 (1939).
20. Cori, C. F., in A symposium on respiratory enzymes, Madison, 186 (1942).
21. Pillai, R. K., *Biochem. J.*, **32**, 1087 (1938)
22. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443 (1942).
23. Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).
24. Schlenk, F., and Schlenk, T., *J. Biol. Chem.*, **141**, 311 (1941).

CANINE CYSTINURIA. URINARY EXCRETION OF CYSTINE FOLLOWING THE ADMINISTRATION OF HOMOCYSTINE, HOMOCYSTEINE, AND SOME DERIVATIVES OF CYSTINE AND CYSTEINE

By W. C. HESS and M. X. SULLIVAN

(From the Chemo-Medical Research Institute, Georgetown University, Washington)

(Received for publication, June 4, 1943)

In human cystinuria, Brand, Cahill, and Harris (1) found a marked increase in cystine excretion following the administration of both methionine and cysteine and, but to a far less extent, with glutathione. Brand, Cahill, and Block (2) also found, in human cystinuria, that homocysteine would cause the excretion of extra urinary cystine, while homocystine would not. The finding with respect to methionine and cysteine has been confirmed by other investigators.

Following the observation that cysteine, homocysteine, and glutathione, all SH compounds, increased urinary cystine in human cystinuria, Brand, Block, Kassell, and Cahill (3) studied the effect of a derivative of cysteine, S-carboxymethylcysteine. This compound, according to these workers, produced a decreased excretion of urinary cystine as measured by the Sullivan method. This they attributed to the formation of thioglycolic acid from the S-carboxymethylcysteine which combines with cysteine to yield a mixed disulfide. Ether extracts of the urine after reduction with zinc and hydrochloric acid gave indications of the presence of thioglycolic acid. Lough, Perilstein, Heinen, and Carter (4) also fed S-carboxymethylcysteine to a cystinuric patient and found some decrease in the excretion of cystine but were unable to detect the presence of thioglycolic acid in the urine after reduction with zinc and hydrochloric acid.

Hess and Sullivan (5) have studied the excretion of urinary cystine in two male, cystinuric Irish terriers. When the dogs were fed a diet containing 10 per cent casein, they excreted approximately 40 mg. of cystine daily. The excretion of cystine could be increased by feeding either methionine or cysteine and the effect in both cases was of the same order of magnitude. As these dogs were excellent experimental subjects whose diets could be completely controlled, it seemed desirable to study further the effect of feeding SH compounds, especially derivatives of cysteine, upon the excretion of urinary cystine.

EXPERIMENTAL

The urinary cystine was determined after precipitation with cuprous chloride as previously described (5). The diet employed was the 10 per

cent casein diet used in the earlier work with the cystinuric dogs (5). The same two dogs, Nos. 32-T and 38-U, were fed, as before, 150 gm. of the diet daily for the first portion of the experiment. These first experiments were done during the winter months; the second series of experiments was performed during July and August and because of the heat the daily ration was reduced to 100 gm. daily for each dog. Subsequently a third series of experiments was done on Dog 38-U with 150 gm. of the diet daily. Table I shows the daily excretion of the principal urinary constituents during the control periods of these three series of experiments. The number of control days in the first and second experimental periods was 8 days each and in the third series, 6 days. The daily values from which the averages are computed are, in all cases, close together.

TABLE I

Average Daily Urinary Excretions at Two Levels of Food Intake of 10 Per Cent Casein Diet

Dog No.	Food intake	Cystine	Nitrogen	Total S	Neutral S
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
32-T	150	0.046	1.97	0.167	0.055
38-U	150	0.043	2.10	0.169	0.052
32-T	100	0.032	1.38	0.120	0.057
38-U	100	0.032	1.36	0.118	0.052
38-U*	150	0.038	1.85	0.167	0.062

* Third series of experiments.

Preparation of Sulfur Compounds—Diglycylcystine was prepared by the method of Fischer and Suzuki (6). The glycylcysteine was prepared by the precipitation of its cuprous mercaptide following the addition of cuprous chloride to a solution of diglycylcystine. This is essentially the Rossouw-Wilken-Jorden method (7) for the preparation of cysteine cuprous mercaptide. The copper compound of glycylcysteine was then treated in a manner similar to that employed by Pirie (8) in the preparation of glycylcysteine. The material was not crystalline but was obtained as a dry, white powder. The peptides were analyzed by hydrolyzing weighed samples and determining the cystine and cysteine respectively in the hydrolysates by the Sullivan method; the yields were practically theoretical in both instances. Nitrogen and sulfur analyses were also performed. Glycylcysteine, theory, N 15.72, S 17.82 per cent; found, N 15.38, S 17.37 per cent; diglycylcystine, theory, N 15.91, S 18.09 per cent; found, N 15.62, S 17.54 per cent.

The thiazolidinecarboxylic acid was prepared from cysteine hydrochloride by the method of Ratner and Clarke (9). It melted at 196–197°. Analysis, theory, N 10.52, S 24.05 per cent; found, N 10.53, S 24.03 per cent.

The homocysteine was prepared from methionine. The methionine was converted into homocysteine thiolactone hydroiodide according to the procedure of Baernstein (10). The thiolactone hydroiodide was then converted into homocysteine according to the procedure of Riegel and du Vigneaud (11). A portion of the homocysteine was oxidized to homocystine by the procedure of Patterson and du Vigneaud (12). Analysis, homocystine, theory, N 10.45, S 23.95; found, N 10.20, S 23.62; homocysteine, theory, N 10.37, S 23.72; found, N 10.20, S 23.42 per cent. Titration of the homocysteine with potassium iodate in the presence of potassium

TABLE II

Total Urinary Excretion for 4 Day Period during Ingestion of Various Derivatives of Cystine and Cysteine

Dog No.	Substance fed	Amount fed	Cystine	Nitrogen	Total S	Neutral S
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
32-T*	Glycylcystine	3.0	0.181	10.2	1.057	0.453
38-U*	Glycylcysteine	3.0	0.199	11.0	0.977	0.314
38-U†	"	3.0	0.192	7.9	1.000	0.281
38-U*	Thiazolidinecarboxylic acid	2.2	0.181	7.7	0.931	0.332
32-T‡	Homocysteine	4.0	0.156	6.0	0.977	0.172
38-U†	"	4.0	0.202	7.8	1.337	0.254
38-U‡	Homocystine	4.0	0.129	5.5	1.052	0.196
32-T‡	Potassium tetracarboxymethylcystine	6.2	0.129	5.8	0.737	0.372
38-U‡	" dicarboxymethylcysteine	6.2	0.130	6 2	0 868	0 554
32-T‡	S-Carboxymethylcysteine	6.0	0.121	5.9	1.268	0.607
38-U‡	"	6.0	0.118	5.9	1 298	0.634

* First series of experiments on a 150 gm. daily food intake.

† Third series of experiments on a 150 gm. daily food intake.

‡ Second series of experiments on a 100 gm. daily food intake.

iodide in 2 per cent hydrochloric acid solution showed the sample to be 97 per cent in the form of homocysteine.

The S-carboxymethylcysteine was prepared by the method of Michaelis and Schubert (13) from cysteine hydrochloride. In the freshly prepared state it melted at 175–176°. The fully dried sample melted sharply at 191–192°. Analysis, theory, N 7.82, S 17.88 per cent; found, N 7.69, S 17.51 per cent.

The tetrapotassium tetracarboxymethylcystine diacetate and the dipotassium dicarboxymethylcysteine acetate were prepared according to the procedure of Michaelis and Schubert (13). Analysis, tetrapotassium tetracarboxymethylcystine diacetate, theory, N 3.76, S 8.60 per cent; found, N 3.73, S 8.25 per cent; dipotassium dicarboxymethylcysteine acetate, theory, N 3.75, S 8.58 per cent; found, N 3.84, S 9.27 per cent. Titration of the

dipotassium dicarboxymethylcysteine acetate with potassium iodate in the presence of potassium iodide in 2 per cent hydrochloric acid solution showed the sample to be 96 per cent in the reduced form.

Table II presents the data on the effect on the urinary constituents of feeding the above compounds to the two dogs. Of the amount of each compound indicated, one-half was fed daily for 2 successive days and the urine was collected for these 2 days and also for the next 2 days to insure complete elimination of any cystine produced by the ingestion of the substance administered.

DISCUSSION

Of all the compounds fed only two produced any extra urinary cystine, homocysteine and glycylcysteine, and these to a limited degree. The 3.0 gm. of glycylcysteine, 100 per cent in the reduced form, correspond to 2.0 gm. of cysteine and produced, as shown in Table III, only 27 mg. and 40 mg. of extra cystine in two separate experimental periods, while the same amount of cysteine, fed as the hydrochloride, to the same dog, No. 38-U, produced 213 mg. of extra cystine (5). 4 gm. of homocysteine, 97 per cent in the reduced form, produced 28 mg. of extra cystine in Dog 32-T and 50 mg. of extra cystine in Dog 38-U. The same amount of homocystine produced no extra cystine in Dog 38-U.

Thiazolidinecarboxylic acid, in which both the sulfur and the nitrogen of the cysteine are bound in a methylene ring, had no effect on urinary cystine excretion. Of the sulfur fed in this compound only 48 per cent was excreted in the urine, about one-half as neutral sulfur and one-half as sulfate sulfur.

Both Brand *et al.* (3) and Lough *et al.* (4) report a decrease in the excretion of urinary cystine when S-carboxymethylcysteine was fed to human cystinurics. When this compound was fed to the two dogs, a slight decrease in extra urinary cystine was produced, 7 mg. in Dog 32-T and 10 mg. in Dog 38-U. The cystine in the dog urine was determined after precipitation by cuprous chloride as earlier detailed (5). If a mixed disulfide of cysteine and thioglycolic acid had been formed following the ingestion of this compound, cuprous chloride should have precipitated the copper mercaptides of both cysteine and of thioglycolic acid. The thioglycolic acid, after removal of the copper, would be titratable by iodine in the Okuda method (14) and the Okuda value on this urine would have been higher than the Sullivan value. This was not the case, and apparently no mixed disulfide was formed in these dogs and excreted in the urine. That no extra cystine was found following the administration of S-carboxymethylcysteine may be in conformity with the fact that this compound does not support the growth of rats on a sulfur-deficient diet (3). While 74 and 77 per cent (Table III) of the sulfur fed was excreted in the urine by the two

dogs, about one-half was excreted as unoxidized sulfur. The low urinary excretion of the sulfur of these various complexes of cystine and cysteine is in agreement with the findings of others (1, 2, 4).

With the two compounds in which only the nitrogen was bound, tetrapotassium tetracarboxymethylcystine diacetate and dipotassium dicarboxymethylcysteine acetate, no extra cystine was produced. On the basis of the compounds fed it is evident that when the sulfur alone, sulfur and nitrogen together, or nitrogen alone of the cysteine is bound to some other group there is no formation of extra urinary cystine. Glycylcysteine, however, gave some extra cystine but only about 13 per cent of that given

TABLE III

Urinary Excretion of Extra Cystine and Sulfur following Ingestion of Various Derivatives of Cystine and Cysteine (4 Day Period)

Dog No.	Substance fed	Cystine	Sulfur		Neutral sulfur
		gm.	gm.	per cent*	gm.
32-T	Glycylcystine	−0.003	0.389	72	0.233
38-U	Glycylcysteine	0.027	0.301	56	0.106
38-U	"	0.040	0.332	62	0.033
38-U	Thiazolidinecarboxylic acid	0.009	0.255	48	0.124
32-T	Homocysteine	0.028	0.497	53	−0.056
38-U	"	0.050	0.669	71	0.006
38-U	Homocystine	0.001	0.580	60	−0.012
32-T	Potassium tetracarboxymethylcystine	0.001	0.257	48	0.144
38-U	" dicarboxymethylcysteine	0 002	0 396	74	0.346
32-T	S-Carboxymethylcysteine	−0.007	0.788	74	0.379
38-U	"	−0.010	0.826	77	0.426

* Per cent of the sulfur of the ingested compound.

by an equivalent amount of cysteine in the first experiment and 19 per cent of that in the second experiment. Whereas, with Dogs 32-T and 38-U, 2 gm. of cysteine gave an average increase in cystine excretion of 145 mg. (5), 8 gm. of homocysteine produced an average extra cystine excretion of only 39 mg.

It may be noted that in the homocysteine experiment, despite the increase in cystine, only 60 per cent of the added sulfur was excreted and, on the average, no extra neutral sulfur. Irregularity in sulfur excretion after various cystine and cysteine complexes were fed was mentioned by Hele and Pirie (15) and by Brand, Cahill, and Block (2). We interpret our findings to the effect that the homocystine and homocysteine absorbed are fully oxidized to sulfate. The experiment with the homocysteine implies also that no homocystine or homocysteine was excreted. In this respect the dogs differ from the human as described by Brand, Cahill, and Block

(2). The lowering of the total sulfur excreted, total sulfur of the basal diet plus total sulfur of the compound fed, was commented on by Brand *et al.* (1) in the case of their human cystinuric. Brand, Cahill, and Block (2) report that only 32 per cent of the homocystine and 66 per cent of the homocysteine fed were found as extra sulfur in the urine.

Brand *et al.* (1) found that glutathione gave a very slight increase in cystine excretion in a human cystinuric, whereas Hele, Hopkins, Lawrie, Leese, Meldrum, and Pirie, according to Hele and Pirie (15), found that a cystinuric could oxidize 3 gm. of glutathione, given by mouth, as readily as cystine.

SUMMARY

When S-carboxymethylcysteine, diglycylcystine, thiazolidinecarboxylic acid, tetrapotassium tetracarboxymethylcystine, or dipotassium dicarboxymethylcysteine was added to the basal diet fed two cystinuric dogs, no extra cystine was excreted, and only a slight increase was observed with homocysteine and glycylcysteine. On the basis of the compounds fed it is evident that when the sulfur alone, sulfur and nitrogen together, or nitrogen alone of the cysteine is bound to some other group there is little, if any, formation of extra urinary cystine. Glycylcysteine gave only 13 to 19 per cent as much extra cystine as did an equivalent amount of cysteine; homocysteine was more effective in that it gave 27 per cent as much extra cystine as was given by an equivalent amount of cysteine.

With S-carboxymethylcysteine there was a slight decrease in the urinary cystine but no evidence of the formation of a mixed disulfide and in the case of homocystine and homocysteine no evidence of their excretion unoxidized.

BIBLIOGRAPHY

1. Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).
2. Brand, E., Cahill, G. F., and Block, R. J., *J Biol. Chem.*, **110**, 399 (1935).
3. Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).
4. Lough, S. A., Perilstein, W. L., Heinen, H. J., and Carter, L. W., *J. Biol. Chem.*, **139**, 487 (1941).
5. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **143**, 545 (1942).
6. Fischer, E., and Suzuki, U., *Ber. chem. Ges.*, **37**, 4575 (1904).
7. Rossouw, S. D., and Wilken-Jorden, T. J., *Biochem. J.*, **29**, 219 (1935).
8. Pirie, N. W., *Biochem. J.*, **25**, 614 (1931).
9. Ratner, S., and Clarke, H. T., *J. Am. Chem. Soc.*, **59**, 200 (1937).
10. Baernstein, H. D., *J. Biol. Chem.*, **106**, 451 (1934).
11. Riegel, B., and du Vigneaud, V., *J. Biol. Chem.*, **112**, 149 (1935-36).
12. Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, **111**, 393 (1935).
13. Michaelis, L., and Schubert, M. P., *J. Biol. Chem.*, **106**, 331 (1934).
14. Okuda, Y., *J. Biochem.*, Japan, **5**, 217 (1925).
15. Hele, T. S., and Pirie, N. W., *Biochem. J.*, **25**, 1095 (1931).

THE CHEMICAL DETERMINATION OF TOCOPHEROLS IN LIVER AND MUSCLE; TOCOPHEROL IN URINE AND FECES

By LEONARD R. HINES and H. A. MATTILL

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

(Received for publication, June 21, 1943)

Only one systematic study of the distribution of tocopherol in animal tissues seems to have been made (1) and this was accomplished by the laborious biological assay. Although direct evidence on the rôle of tocopherol in cellular metabolism is thus far confined to its action in muscle (2, 3), its presence in other tissues, whether as functional or as stored material, calls for a reliable chemical method of assay. When the technique of Devlin and Mattill (4) for the determination of tocopherol in muscle was applied to liver tissue, its shortcomings became immediately obvious. This paper describes the modifications by which that method appears to have been improved and made adequate for use with liver, urine, and feces.

With extracts of liver tissue prepared as described for muscle (4), unreasonably high readings were obtained at the end of 10 minutes and these were still increasing after 2 hours. The gradual increase in color production in the presence of cholesterol was confirmed both in a benzene solution of tocopherol and cholesterol and in liver extracts. Such extracts even after adsorption on florisil and treatment by the acid-alkali technique of Parker and McFarlane (5) still contained cholesterol. On the other hand, when a solution of tocopherol and cholesterol in benzene was adsorbed on florisil, cholesterol was completely removed and none of the tocopherol was adsorbed; something in the complex extract obtained from liver interfered with the complete removal of cholesterol by adsorption.

This substance appeared to be vitamin A; when a solution of cholesterol, tocopherol, and a vitamin A concentrate[1] in benzene was similarly adsorbed, a considerable amount of cholesterol remained in the solution. The presence of vitamin A in the liver extracts, even after adsorption on florisil, was indicated by the transient purple color (6) appearing upon the addition of 85 per cent sulfuric acid in the acid-alkali treatment. Vitamin A was apparently destroyed by this procedure, because when a second acid-alkali treatment was applied to such mixtures, the intense purple color which appeared in the first acid treatment was entirely absent.

[1] Courtesy of Distillation Products, Inc., and Dr. Hickman.

When liver extracts, after a single acid-alkali treatment, were passed through two columns of florisil, stable colorimeter readings were obtained. Such extracts still contained some cholesterol, but small amounts apparently do not seriously derange the course of color development. The reason for the incomplete adsorption of cholesterol under these conditions remains to be discovered.

In two respects, therefore, the original method was altered; the order of adsorption on florisil and acid-alkali treatment was reversed and a second adsorption on florisil was introduced. With these modifications liver extracts gave maximum color intensity at the end of either 10 or 20 minutes. Readings at 20 minutes were usually not more than 3 or 4 units greater than at 10 minutes, and since pure tocopherol solutions produce maximum color development at 10 minutes, all readings were made at that time. With muscle extracts, the reversal of the order of acid-alkali treatment and a single adsorption were sufficient; extracts so prepared gave maximum color development at the end of 10 minutes as against 30 minute readings heretofore used.

The method finally employed, embodying these and other minor modifications, was as follows: Liver tissue (8 to 10 gm.) was shredded in 60 ml. of Skellysolve B[2] (SSB) and 70 ml. of redistilled absolute alcohol in an ice-cooled Waring blendor. The insoluble tissue residue, after centrifugation, was subjected to three similar extractions. The four clear supernatant liquids were pooled in a 2 liter separatory funnel and partitioned into an alcohol-water and a petroleum ether phase by the addition of 600 ml. of water. The separatory funnel was gently whirled, sufficient alcohol was added to break the emulsion, and the petroleum ether fraction usually cleared within a few minutes. The alcohol-water fraction was then extracted three times with 150 ml. of SSB. The petroleum ether fractions were pooled and distilled under nitrogen at reduced pressure on a hot water bath. The clear residual oil was taken up in 25 ml. of purified Skellysolve E[2] (SSE). To 10 ml. of this solution in a glass-stoppered centrifuge tube, 2 ml. of 85 per cent sulfuric acid were added, and after shaking, the tube was centrifuged for 5 minutes. As much of the clear supernatant as possible was then drawn off and transferred to a second glass-stoppered centrifuge tube, 5 ml. of 1 per cent potassium hydroxide were added, and, after shaking, the tube was centrifuged for 10 minutes. 7 ml. of the clear supernatant were drawn off and evaporated to dryness under nitrogen on a boiling water bath. The residue was taken up in 7 ml. of C.P. benzene, 6 ml. of which were passed through a 70 × 12 mm. column of florisil through

[2] Purified by shaking three times with concentrated sulfuric acid, and washing with water, dilute sodium hydroxide, and water; dried over anhydrous calcium chloride and distilled.

which 10 ml. of benzene had previously been passed to moisten the column. The column was washed with 3 to 5 ml. portions of benzene, and the resultant solution evaporated to dryness under nitrogen on the water bath. The residue, containing the tocopherols, was taken up in 6 ml. of benzene, 5 ml. of which were passed through a second similar column of florisil. After again being washed and evaporated to dryness, the residue was taken up in 5 ml. of SSE, 20 ml. of the modified α,α'-bipyridine reagent[3] were added, and the resultant color read at the end of 10 minutes in a Klett-Summerson photoelectric colorimeter with a No. 52 light filter. Tocopherol values were then read on a calibration curve.

When known amounts of tocopherol were added to the blendor along with liver tissue, other portions of which were analyzed without such additions, the amounts of tocopherol recovered corresponded closely to those expected, as shown in Table I. Such figures for recovery are not completely

TABLE I

Recovery of α-Tocopherol Added to Liver Tissue

Tocopherol content of sample (*a*)	Tocopherol added (*b*)	Total tocopherol found (*c*)	Recovery, $\frac{(c)-(a)}{(b)} \times 100$
γ	γ	γ	*per cent*
103.4	100.0	200.0	96.6
67.0	100.0	170.0	103.0
111.5	150.0	255.0	96.3
Average recovery			98.6

valid evidence for the accuracy of the method, but they are certainly favorable.

The variations in tocopherol content of muscle and liver under various dietary régimes, as determined by this method, are recorded in Table II. It is apparent, as first shown by Mason (1), that the liver of the rat and rabbit may store tocopherol when the vitamin is generously supplied in the diet. The muscles of both species also appear to store it but to a less extent. The present figures for the tocopherol content of rat muscle are somewhat lower than the values reported by Devlin and Mattill (4). This difference may be related to the changes in procedure; those readings were made at the end of 30 minutes, these at the end of 10 minutes. Since pure tocopherol solutions and most of our tissue extracts attain maximum color development at the end of 10 minutes, it is not unlikely that the color development taking place after this time may be due to the oxidation

[3] 250 mg. of ferric chloride hexahydrate and 500 mg. of α,α'-bipyridine made up to 1 liter with glacial acetic acid.

of substances unrelated to tocopherol, substances which by the revised technique are more completely removed from the extracts.

These new and lower figures for so called normal animals still further reduce the "minimum fertility dose" of muscle tissue but the adequacy of tocopherol in some commercial animal feeds has been questioned (1); recently in a neighboring laboratory,[4] male rats raised on a dog biscuit diet were found to be sterile, with a histological picture of the testes typical of vitamin E-deficient rats. This may also explain the similarity of the liver content of normal and deficient animals, both rats and rabbits. By

TABLE II

Effect of Diet on Tocopherol Content of Liver and Muscle

The results are expressed in mg. of tocopherol per kilo of tissue.

	High vitamin E*		Normal†		Vitamin E-deficient	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
Rats; males	52.8	15.6	20.1	5.6	24.3	3.9
	47.2	9.4	19.6	8.7	19.5	6.4
	26.8	10.7	26.5	8.3	23.5	5.4
					24.1	3.2
Average . .	42.3	11.9	22.1	7.5	22.6	4.8
Rabbits	86 8	28.1	8.5	10 6	8.3	3.5
			9 8	6.5	8.9	8.0
				7.0	11.2	5.5
Average			9.2	8.0	9.4	5.7

* Ground dog chow supplemented with vitamin E concentrates so that each rat received an estimated 100 mg. of tocopherol daily. The rabbits received a vitamin E-deficient diet supplemented with synthetic *dl*-α-tocopherol so that each animal received an estimated 18 mg. daily.

† Dog chow or rabbit chow.

the revised technique, the tocopherol content of muscle from animals on an enriched diet was also lower than that heretofore reported (4). As indicated below, much of the tocopherol in such diets may not be absorbed.

Some importance may attach to the relatively high content of tocopherol in rat liver as compared with rat muscle under all conditions of feeding, in contrast to the much smaller differences between liver and muscle of rabbits under vitamin E deprivation or on supposedly normal diets. Abundant hepatic storage capacity might explain the protracted vitamin E deprivation which rats may undergo without demonstrating the critical shortage to which rabbits succumb in much shorter time.

Evidence for the importance of tocopherol in muscle metabolism has

[4] Fugo, N. W., personal communication.

recently been reviewed (7) and, since tocopherol phosphate, but not tocopherol itself, altered the course of oxidation *in vitro* (2), it is conceivable that in animal tissues the vitamin might occur bound in such a fashion that simple extraction with organic solvents does not remove it. Muscle residue, after four extractions with SSB and alcohol in the usual manner, was twice extracted with these same solvents containing 5 ml. of concentrated hydrochloric acid. The two resulting extracts were pooled and analyzed for tocopherol by the original method (4). The results from muscle of three normal rats indicated that 4.5, 2.9, and 3.2 mg. of additional tocopherol per kilo of muscle had been thus released, from three rats on a high tocopherol diet 2.1, 1.7, and 2.1 mg., and from a single vitamin E-deficient rat 2.1 mg. Perhaps these figures represent some reacting substance other than tocopherol but the requisite properties as to solubility, adsorption, and survival after acid-alkali treatment leave little choice for speculation. Among the agencies concerned in biological oxidations are several phosphorylated substances bound to protein.

Tocopherol is readily oxidized to tocopherylquinone *in vitro* (8) and if this oxidation occurs in animal tissues, the process is not reversible (9). The finding of tocopherylquinone in dog plasma (10) suggested that the utilization of tocopherol might take place in this manner. The cyclization procedure of Tishler and Wendler (11) provided a technique applicable to tissue extracts, by which the presence of tocopherylquinone could be demonstrated. When the original extracts of muscle or liver were subjected to cyclization prior to acid-alkali treatment and double adsorption, it was possible to find 95 per cent of the tocopherol present in non-cyclized extracts, but not more. This was true whether the tissues came from rats on stock diets or from rats on diets high or deficient in tocopherol.

Information concerning the excretion of tocopherol is limited to the reports of McArthur and Watson (12) who were unable to demonstrate its presence in cow urine and of Cuthbertson and coworkers (13), who, by a spectroscopic method, were unable to detect it in liver extracts of rats on diets high in tocopherol. The present method seemed to offer a means of answering this question.

The urine of two rats, receiving an estimated 100 mg. of tocopherol daily for 2 months previously and during the test, was collected for 5 days and pooled. On the chance that tocopherol might be excreted in conjugated form, the urine was acidified to pH 1 and refluxed for 4 hours; on cooling, it was extracted four times with purified SSB, the solvent was distilled off under nitrogen at reduced pressure, and the residue was analyzed for tocopherol. In agreement with previously reported results, no tocopherol was found, even under conditions of excessively high intake. Application of the cyclization procedure (11) to the residue and subsequent analysis for tocopherol also gave completely negative results, indicating that tocoph-

erylquinone is not excreted in the urine of rats receiving massive doses of tocopherol.

The feces of these rats were examined for tocopherol, and although quantitative observations were not made, the results indicated that very large amounts were present, whereas feces of rats on a stock diet gave completely negative results.

Since liver might be expected to offer a greater amount and variety of interfering substances than any other animal tissues, the method described may have general application. This has not been demonstrated and, for the present, the development and application of this method may not be continued; it is hoped that others interested may find it reliable and may improve upon it.

SUMMARY

With further modifications of the method based on the iron-α,α'-bipyridine color reaction, the tocopherols in liver tissue and in urine and feces have been determined.

The tocopherol content of rat and rabbit liver tissue from animals on diets high in tocopherol, commercial laboratory chow, and on vitamin E-deficient diets averaged 42.3, 22.1, 22.6, and 86.8, 9.2, and 9.4 mg. per kilo respectively. Muscle tissue of the same animals averaged 11.9, 7.5, 4.8, and 28.1, 8.0, and 5.7 mg. per kilo respectively. No tocopherol was found in the urine of rats on high tocopherol intake, nor was evidence obtained for the presence of tocopherylquinone in liver or muscle or its excretion in the urine, although considerable tocopherol was found in the feces under those conditions. Evidence was obtained indicating that not all the tocopherol is removed from tissues by simple extraction with organic solvents.

The possible significance of these findings is indicated.

BIBLIOGRAPHY

1. Mason, K. E., *J. Nutrition*, **23**, 71 (1942).
2. Houchin, O. B., *J. Biol. Chem.*, **146**, 313 (1942).
3. Kaunitz, H., and Pappenheimer, A. M., *Am. J. Physiol.*, **138**, 328 (1943).
4. Devlin, H. B., and Mattill, H. A., *J. Biol. Chem.*, **146**, 123 (1942).
5. Parker, W. E., and McFarlane, W. D., *Canad. J. Research, Sect. B*, **18**, 405 (1940).
6. Fearon, W. R., *Biochem. J.*, **19**, 888 (1925).
7. Pappenheimer, A. M., *Physiol. Rev.*, **23**, 37 (1943).
8. Karrer, P., and Geiger, A., *Helv. chim. acta*, **23**, 455 (1940).
9. Golumbic, C., and Mattill, H. A., *J. Biol. Chem.*, **134**, 535 (1940).
10. Scudi, J. V., and Buhs, R. P., *J. Biol. Chem.*, **146**, 1 (1942).
11. Tishler, M., and Wendler, N. L., *J. Am. Chem. Soc.*, **63**, 1532 (1941).
12. McArthur, C. S., and Watson, E. M., *Canad. Chem. and Process Ind.*, **23**, 350 (1939).
13. Cuthbertson, W. F. J., Ridgeway, R. R., and Drummond, J. C., *Biochem. J.*, **34**, 34 (1940).

THE RECOVERY OF CRYSTALLINE THYROXINE FROM IODINATED CASEIN*

By E. P. REINEKE and C. W. TURNER

(From the Department of Dairy Husbandry, University of Missouri, Columbia)

(Received for publication, May 27, 1943)

In previous reports from our laboratory, it has been shown that the thyroidal potency of iodinated proteins is determined by the extent of iodination and also the conditions maintained in the reaction medium during and subsequent to the iodination. Other factors being held constant, maximum activity is obtained (1, 2) when the iodine is limited to the amount required for substitution of 2 atoms per mole of tyrosine in the protein. If the iodine input is held constant at this optimal level, and the temperature is elevated to approximately 70° during the subsequent incubation period of 18 to 20 hours (3), products with surprisingly high thyroidal potency are obtained. Assayed by their metabolic effect when given orally to guinea pigs, these preparations show 3 to 4 per cent of the effect of pure thyroxine administered in the same manner. When injected into tadpoles, they produce a metamorphosis response equivalent to 8 to 11 per cent of that produced by thyroxine.

Ludwig and von Mutzenbecher (4) and Harington and Rivers (5) first reported that thyroxine could be isolated from iodinated proteins that had been prepared under narrowly limited conditions.

In view of the high biological activity of iodinated proteins that were prepared under the optimal conditions established by our previous studies, it appeared likely that a much higher yield of thyroxine should be obtained from them than had previously been reported. Data that show this to be the case have now been obtained and are presented in this report.

EXPERIMENTAL

The iodinated casein used for the hydrolysis and recovery of thyroxine was taken from a large batch that had been prepared under the optimal conditions described previously (3). A commercial grade of acid-precipitated casein was suspended in distilled water containing 0.7 per cent sodium bicarbonate, in the ratio of 100 gm. of casein to 4 liters of solution. The solution was heated to 40° with continuous stirring, and finely powdered iodine was then added intermittently over a period of about 4 hours in the ratio of 17.6 gm. of iodine per 100 gm. of casein. When all the iodine had

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series, No. 895.

been combined with the casein, the temperature was increased to 70°, and the mixture was held at this temperature, with strong, continuous stirring for 20 hours.

The iodinated protein was precipitated by the addition of dilute hydrochloric acid to a pH of about 4.0. The liquid was siphoned off, and the precipitate was partially purified by resuspending it with the aid of sodium hydroxide and precipitation with dilute hydrochloric acid. After this process was repeated twice, the iodinated protein was recovered by filtration, dried, and ground to a fine powder.

Thyroxine was recovered by a procedure similar to that described by Ludwig and von Mutzenbecher (4). 100 gm. of the iodinated protein, prepared as outlined above, were mixed with 640 ml. of distilled water, and placed in a digestion flask. 320 gm. of $Ba(OH)_2 \cdot 8H_2O$ were added, and the solution was agitated occasionally while being heated until the barium hydroxide was all dissolved. The solution was then boiled gently under a reflux for 20 hours. The precipitate of barium salts was removed by running the hot solution through a suction filter. The precipitate (A) and filtrate (B) were then treated separately as follows: Precipitate A was treated with dilute HCl, with vigorous stirring until the reaction became slightly acid to Congo red, filtered with suction, and washed with dilute acetic acid. Filtrate B was cooled, and the crystalline $Ba(OH)_2$ was removed by filtration, redissolved, and crystallized again, and the second filtrate was combined with the first. The combined filtrate was acidified with dilute HCl to pH 4.5 to 5.0, whereupon a light colored precipitate settled out. The precipitate was recovered by centrifuging, washed several times with dilute acetic acid, dried, and added to Precipitate A. After being dried, the combined precipitate was extracted with three changes of ether, and then dissolved in 0.1 N NaOH solution containing a small amount of sodium sulfate for removal of the barium, and heated to boiling. The barium sulfate was removed by centrifuging, this process being repeated until a clear amber-colored supernatant solution was obtained. The solution was heated to 90° and cautiously acidified with dilute sulfuric acid until a granular precipitate formed (pH 5.0). The precipitate was recovered by filtration, washed with dilute acetic acid, and dried *in vacuo* over magnesium perchlorate. In two different separations yields of 3.14 and 3.40 gm. of acid-insoluble substance were obtained from 100 gm. of protein. In the first trial, all of the acid-insoluble fraction was used for the isolation of thyroxine; in the second a small amount was retained for analysis and assay.

The acid-insoluble substance was dissolved in the minimum amount of 0.1 N sodium carbonate solution, centrifuged to remove traces of undissolved, dark colored substance, and then chilled to 0°. A heavy white

precipitate of the monosodium salt of thyroxine formed. The precipitate was collected by centrifuging, and purified by crystallizing several times from 0.1 N Na_2CO_3 solution. Additional amounts of the monosodium salt were obtained by successively concentrating the mother liquors by boiling, and purifying the white precipitate that formed upon cooling.

The monosodium salt was taken up in 70 per cent alkaline alcohol, heated to boiling, and acidified with acetic acid. Thyroxine separated immediately in the characteristic bundles of microscopic needles. It was purified by a second crystallization from hot 0.1 N sodium carbonate, and finally from boiling alkaline alcohol. In one instance a yield of 424 mg. of crystalline thyroxine was obtained by this process from 100 gm. of iodinated casein. In the second trial a yield equivalent to 385 mg. was obtained.

Analyses and Biological Assays—The preparations were analyzed for iodine by the method of Kendall as described by Harington (6). Assays on tadpoles were conducted as described previously (3). For the metabolic assays, the substances to be tested were weighed accurately, dissolved in distilled water with the aid of a small amount of sodium hydroxide, and administered orally in proportion to body weight. Beginning on the 6th day of dosage, the carbon dioxide production was determined gravimetrically in a modified Haldane type of open circuit respiration apparatus. All feed was removed at 9.00 a.m., and the metabolism determinations were made for a period of 1 hour on each animal during the afternoon. As a measure of the amount of metabolic stimulation, the percentage increase in carbon dioxide production above the normal control values obtained under the same conditions was calculated.

The iodinated casein prepared for these experiments contained 7.5 per cent of iodine. When administered orally to four guinea pigs at the rate of 0.4 mg. per 100 gm. of body weight daily, the carbon dioxide output was increased by an average of 38.6 per cent during 4 days from the 6th to 9th day of dosage. By comparison with a reference curve that was established by administration of thyroxine to guinea pigs at various levels under similar conditions, it was determined that this response is equivalent to 12 γ of thyroxine. Therefore, the iodinated casein actually showed 3 per cent of the potency of thyroxine, if they are compared on an equal weight basis. In three different assays on tadpoles the iodinated casein showed an average of 8.6 per cent of the potency of thyroxine, or approximately 2.8 times the value obtained on guinea pigs. This agrees with the relationship between these methods of assay established previously (3).

Data on the recovery of crystalline thyroxine from this iodinated protein are given in condensed form in Table I. The high thyroidal potency of the iodinated casein prepared by our method is borne out by the increased yields of thyroxine obtained from it. Our recovery of crystalline thyroxine

amounted to 0.424 and 0.385 per cent, respectively, in two separate trials, as compared to a yield of approximately 0.100 per cent reported by Ludwig and von Mutzenbecher (4) and Harington and Rivers (5).

It is of considerable interest to attempt to correlate the yield of crystalline thyroxine obtained with the thyroidal potency of the original iodinated casein. Our maximum yield of 0.424 per cent would appear at first hand to account for only about one-seventh of the potency of the iodinated casein, since this preparation showed 3.0 per cent of the thyroidal effect of thyrox-

TABLE I

Yield, Analysis, and Metabolic Assay of Thyroxine and Thyroxine Concentrate Obtained from Iodinated Casein

Preparation	Yield	Iodine content	Metabolic assay							
			Assay No.	No. of guinea pigs	Dosage	Average gain in CO_2 output*				
						Trial 1	Trial 2	Trial 3	Trial 4	Combined average
	per cent	*per cent*			*γ per 100 gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Hydrolysis 1, acid-insoluble substance	3.14									
Thyroxine	0.424	64.8 65.0	1	4	8	29.4	24.5	32.7	31.4	29.5
Hydrolysis 2, acid-insoluble substance	3.40	40.12 40.12	1	4	13	31.3	30.8	29.2	30.4	30.4
Thyroxine	0.385	63.6 63.6	2	4	10	34.7	25.0			29.8
Assay standard, synthetic thyroxine		65.0	2	4	10	30.9	28.5			29.7

* The CO_2 production was determined between the 6th and the 10th day after oral dosage of the test substance was begun, and computed as the percentage increase above normal control values obtained under the same conditions.

ine when tested on guinea pigs. We have been able to establish recently, however, that the thyroxine as it occurs in the iodinated casein molecule actually exists in the levorotatory form. (See the paper following.) Our own data, as well as the report of Foster *et al.* (7), indicate that *l*-thyroxine has twice the calorigenic effect of the usual *dl* mixture obtained after hydrolysis of thyroxine-containing proteins with alkali. The apparent thyroidal effect of the protein should therefore be divided by 2 for comparison with the yield, since it was assayed against racemic thyroxine. On this basis the actual yield of crystalline thyroxine is approximately 28 per cent of the value indicated biologically. It is well recognized, furthermore, that

the isolation of thyroxine is far from quantitative, owing both to destruction during hydrolysis, and to unavoidable losses during the process of purification.

For this reason the crude acid-insoluble substance obtained during the purification is believed to represent more nearly the actual amount of thyroxine present. In the one experiment in which a portion of this fraction was withheld for analysis and assay, a yield of 3.4 gm. was obtained from 100 gm. of starting material. Since the iodine content was 40.1 per cent, this would be equivalent to 2 per cent thyroxine in the iodinated protein. 13 γ of this substance would be equivalent in iodine content to 8 γ of thyroxine. When these amounts of acid-insoluble substance and thyroxine, respectively, were administered to groups of guinea pigs on which experiments were run side by side in the same assay, the same average metabolic response was obtained. If all of the metabolic effect of the acid-insoluble substance is assumed to be due to thyroxine, then all of the activity of the iodinated protein as shown by the assays on guinea pigs can be accounted for by its thyroxine content.

The assay value of the iodinated protein is equivalent to 3.0 per cent *dl*-thyroxine or about 1.5 per cent of the *l* form. Thus the value of 2 per cent estimated from the acid-insoluble fraction appears to indicate an excessive recovery. The discrepancy between the two figures is within the range of experimental error of ± 20 per cent on each assay, however.

In all of our investigations of iodinated proteins covering a wide range of thyroidal activity, the assay results with tadpoles have run parallel with the results on guinea pigs, but have shown absolute values 2.7 to 2.8 times as great when expressed on the thyroxine basis. Although the values obtained on guinea pigs can be accounted for by the amount of thyroxine apparently present, further investigation is required before the uniformly higher values on tadpoles can be explained.

By reference to Table I, it will be seen that both samples of thyroxine obtained from iodinated casein exerted a metabolic effect that, within the limits of experimental error, was identical with that of synthetic thyroxine. The iodine contents were 65.0 and 63.6 per cent respectively, as compared to 65 per cent in the synthetic standard. The melting point, when the material was heated at the rate of 3° per minute, was 230–232°.

Spectrographic absorption curves obtained on a sample of the thyroxine obtained from casein in comparison with synthetic thyroxine are shown in Fig. 1. Both samples were dissolved in alkaline butyl alcohol in a concentration of 0.2 mg. per ml. The absorption of light in the ultraviolet region was determined in a Hilger medium quartz spectrograph, together with a Hilger-Spekker spectrophotometer, and a 1 cm. absorption cell. The light source was an iron-tungsten spark.

As the concentration was the same in both preparations, the extinction (log I_0/I) is plotted directly against the wave-length in Å. It will be observed that the absorption of the two compounds is practically identical throughout the range from 2600 to 4000 Å. This, together with the

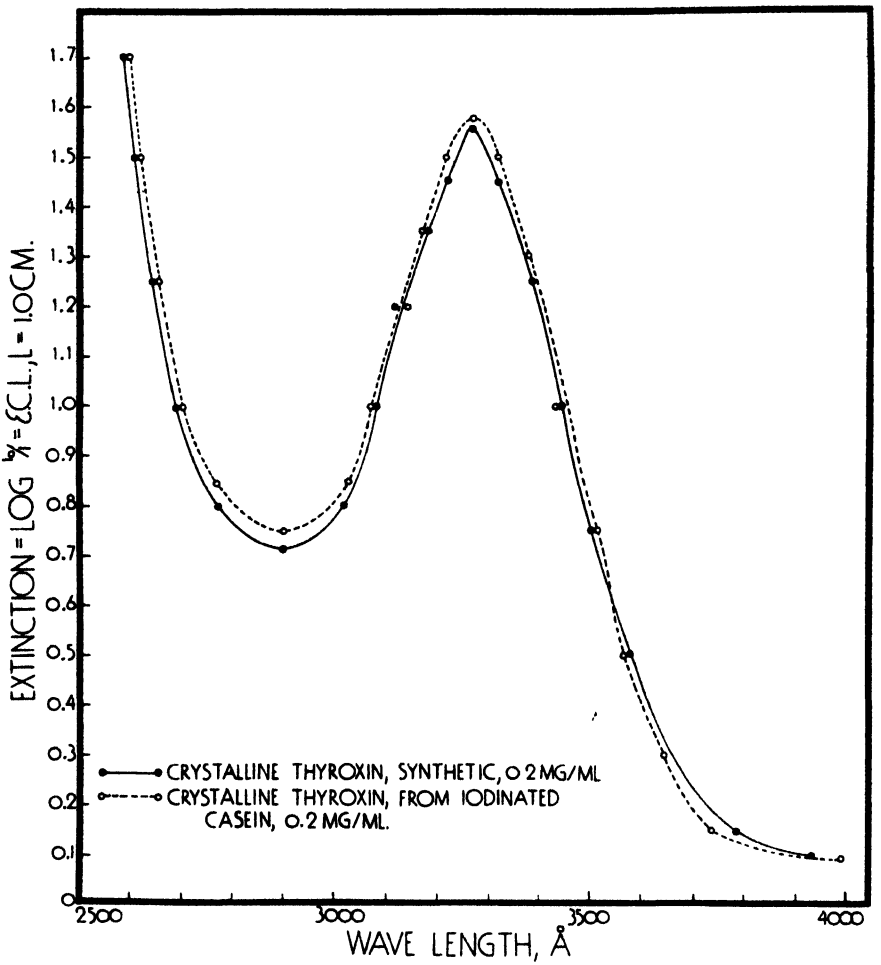


FIG. 1. Spectrographic absorption curves for thyroxine obtained from casein and for synthetic thyroxine.

analytical data and biological assays, can leave no doubt that the crystalline substance isolated in high yield from iodinated casein is actually thyroxine.

SUMMARY

An iodinated casein was prepared and crystalline thyroxine was isolated from it subsequent to hydrolysis with barium hydroxide in order to deter-

mine whether or not the yield of thyroxine could be correlated with the thyroidal activity of the iodinated protein.

In two trials, yields of 424 and 385 mg. of crystalline thyroxine were obtained from 100 gm. of starting material. The iodinated protein itself showed thyroidal activity equivalent to 3 per cent that of racemic thyroxine. Since thyroxine is apparently formed in the protein in only the active levo form, however, the highest yield obtained would account for 28 per cent of the activity of the original iodinated protein.

An impure acid-insoluble substance containing 40.1 per cent iodine was obtained in a yield of 3.4 per cent. A biological assay showed it to have activity equivalent to thyroxine when given at the same iodine level. If all the activity of this substance is assumed to be due to thyroxine, the thyroidal activity of the iodinated casein, as measured by the guinea pig assay, would be completely accounted for.

The identity of the thyroxine obtained was verified by its iodine content, melting point, spectrographic absorption, and biological assay.

The authors wish to acknowledge their appreciation to Dr. V. R. Ells, Spectrographic Service, University of Missouri, for the spectrographic determinations and to Vernon R. Cupps, student assistant, for assistance with the biological assays.

BIBLIOGRAPHY

1. Reineke, E. P., Williamson, M. B., and Turner, C. W., *J. Biol. Chem.*, **143**, 285 (1942).
2. Reineke, E. P., Williamson, M. B., and Turner, C. W., *J. Biol. Chem.*, **147**, 115 (1943).
3. Reineke, E. P., and Turner, C. W., *Missouri Agric. Exp. Stat., Research Bull.* **355** (1942).
4. Ludwig, W., and von Mutzenbecher, P., *Z. physiol. Chem.*, **258**, 195 (1939).
5. Harington, C. R., and Rivers, R. V P., *Nature*, **144**, 205 (1939).
6. Harington, C. R., The thyroid gland; its chemistry and physiology, London, 192 (1933).
7 Foster, G. L., Palmer, W. W., and Leland, J. P., *J. Biol Chem.*, **115**, 467 (1936)

THE RECOVERY OF *l*-THYROXINE FROM IODINATED CASEIN BY DIRECT HYDROLYSIS WITH ACID*

By E. P. REINEKE and C. W. TURNER

(From the Department of Dairy Husbandry, University of Missouri, Columbia)

(Received for publication, May 27, 1943)

In the preceding report it was shown that iodinated casein prepared under optimal conditions will yield somewhat more than 0.4 per cent of crystalline thyroxine subsequent to hydrolysis with barium hydroxide. Since hydrolysis with strong alkali results in racemization of amino acids, however, the thyroxine obtained is a *dl* mixture, and provides no evidence as to whether or not it was originally formed in the protein in an optically active state.

The only method used successfully thus far for the isolation of *l*-thyroxine from thyroid gland substance has involved stepwise hydrolysis with proteolytic enzymes and successive purification of the hydrolysates. Harington and Salter (1) first reported the recovery of *l*-thyroxine from thyroid after intensive digestion with pepsin and trypsin. Foster *et al.* (2) used a similar method, but employed 2 N sulfuric acid to complete the hydrolysis.

The early investigations of Baumann (3) and others indicated that iodothyrin, prepared by hydrolysis of thyroid with 10 per cent sulfuric acid, contained a physiologically active substance. However, in later experiments discussed by Kendall (4) all attempts to isolate thyroxine from iodothyrin resulted in failure.

Lerman and Salter (5) reported the stepwise separation of iodinated serum protein into T and D fractions by hydrolysis with pepsin and trypsin in a manner similar to that observed previously with thyroglobulin (6). However, attempts to isolate thyroxine were unsuccessful. At the same time that they announced the isolation of crystalline thyroxine from iodinated casein subsequent to hydrolysis with barium hydroxide, Ludwig and von Mutzenbecher (7) reported that they were unable to recover a crystalline product after hydrolysis with either sulfuric acid or proteolytic enzymes.

As is shown in the preceding paper, we have been able to recover more than 0.4 per cent of crystalline thyroxine from iodinated casein prepared under optimal conditions, by use of the usual alkaline hydrolysis. During the course of this work, it occurred to one of us that, if the iodinated protein were hydrolyzed with an acid in the presence of a solvent, the hydrolysis

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series, No. 896.

might go to completion without the destruction of thyroxine that apparently occurred in all of the previous work. By use of this technique, it has been found possible to obtain crystalline *l*-thyroxine from iodinated casein in satisfactory yield by a relatively simple method.

EXPERIMENTAL

As a preliminary, the miscibility of *n*-butyl alcohol with various concentrations of sulfuric acid solution was tested. It was observed that, when an aqueous solution containing 18 to 32 per cent sulfuric acid was mixed with an equal volume of *n*-butyl alcohol, the butyl alcohol would separate into a clear cut layer. When the 18 per cent acid solution was heated, little change in the distribution of the layers occurred. When the 32 per cent acid solution was heated, however, the butyl alcohol and sulfuric acid became completely miscible, although they separated again when the solution was cooled.

Hydrolysis with Sulfuric Acid—When a small amount of iodinated casein was placed in a solution containing 18 per cent of sulfuric acid together with an equal volume of butyl alcohol and heated to boiling, the hydrolytic products were largely taken up by the butyl alcohol layer within the course of a few minutes, even though the hydrolysis was far from complete. Therefore in subsequent work a 32 per cent sulfuric acid solution was used in order to obtain more complete hydrolysis.

In the first attempt by this procedure, 25 gm. of iodinated casein were hydrolyzed by heating for 14 hours in a mixture containing equal parts of *n*-butyl alcohol and 32 per cent sulfuric acid. After successive purification of the hydrolysate, 16 mg. of a crystalline substance that was identical with thyroxine in its appearance and general properties were obtained. Accordingly a larger amount of iodinated casein taken from an entirely different lot was worked up in order to obtain a yield sufficient to identify the compound more fully. The following procedure was used.

The iodinated casein was prepared exactly as described in the preceding paper. 600 gm. were mixed with 3 liters of *n*-butyl alcohol, and 3 liters of an aqueous solution containing 32 per cent sulfuric acid were added. The mixture was refluxed on a boiling water bath until the biuret test became negative, a period of 13 hours. After cooling, 6 liters of distilled water were added and mixed thoroughly.

The solution was then placed in a large separatory funnel, and the aqueous layer was drawn off and discarded. In order to remove a considerable amount of alkali-soluble, dark colored impurity, the butyl alcohol layer was extracted three times in succession with 3 liter portions of a solution containing 16 per cent of sodium hydroxide and 5 per cent of sodium carbonate, the aqueous layer being discarded each time. In order to avoid racemization, the extractions were carried out as rapidly as possible, and

immediately after the last extraction the butyl alcohol portion was adjusted to pH 4.5 to 5.0 by the addition of dilute hydrochloric acid.

The butyl alcohol was removed by vacuum distillation with gentle warming in a water bath. The residue was dissolved in approximately 5 liters of distilled water with the aid of ammonium hydroxide, heated to 60°, and warm barium hydroxide solution was added until a flocculent precipitate formed. The precipitate of barium salts (Precipitate A) was filtered off quickly on a Buchner funnel, leaving a clear, straw-colored filtrate (B). When the filtrate was acidified to pH 4.5 to 5.0 with dilute acetic acid, a light colored precipitate settled out. Precipitate A was decomposed by making it slightly acid to Congo red with hydrochloric acid, and the acid-insoluble material was recovered by filtration, dissolved in 1 liter of distilled water with the aid of ammonia, heated to 60°, and treated with a slight excess of warm barium hydroxide solution as before. After the barium salts were filtered off, a second small quantity of acid-insoluble substance was recovered from the filtrate. This process was repeated twice more, a small amount of acid-insoluble material being recovered each time.

The combined precipitates were dissolved in about 800 ml. of distilled water with the aid of ammonia, warmed to 60°, and a few ml. of saturated ammonium sulfate solution were added. The precipitate of barium sulfate was removed by centrifuging. The clear, amber-colored solution that remained was heated to 85°, and cautiously acidified with dilute sulfuric acid solution to the point of maximum precipitation (pH 5.0). The granular precipitate was collected by filtering while hot, and then washed with dilute acetic acid.

After the material was dried *in vacuo* over magnesium perchlorate, a yield of 7.2 gm. of acid-insoluble precipitate containing 42 per cent of iodine was obtained. Of this precipitate 7.1 gm. were dissolved in 300 ml. of hot 0.1 N sodium carbonate and centrifuged while hot to remove a small amount of undissolved matter. When this solution was cooled to 0°, a heavy micro crystalline precipitate of the monosodium salt of thyroxine settled out. The thyroxine was purified as described in the preceding paper, and was finally crystallized from alkaline alcohol as the free amino acid. A total yield equivalent to 595 mg. of crystalline thyroxine was obtained by this method from 600 gm. of starting material. In addition, a fraction that was estimated to contain approximately 100 mg. of thyroxine was lost through breakage of a centrifuge tube. The probable yield was thus well over 0.1 per cent of the original iodinated casein.

The crystalline compound was found to contain 65.1 per cent iodine. The melting point, when the compound was heated at the rate of 3° per minute, was 236–238°, as compared to a melting point of 230–232° obtained with *dl*-thyroxine by the same method.

Optical Rotation—The optical rotation was determined in a highly sensi-

tive Schmidt and Haensch polariscope, with a sodium vapor lamp as light source. 210 mg. of the crystalline thyroxine were dissolved in 5 ml. of a solution consisting of 24 gm. of 0.5 N sodium hydroxide mixed with 56 gm. of absolute ethyl alcohol. The observed rotation was −0.176°, or $[\alpha]_D = -4.2°$. The specific rotation of the thyroxine obtained by acid hydrolysis of iodinated casein thus agrees fairly well with the value of −4.4° reported by Foster *et al.* (2) for thyroxine recovered by the enzymatic hydrolysis of thyroid tissue, but is slightly higher than the value of −3.8° reported by Harington and Salter (1) for the product obtained both by resolution of synthetic thyroxine and by isolation from thyroid. It should be noted that the latter workers used a mercury green light for the polariscope determination, whereas our data and those of Foster *et al.* were obtained with sodium light.

Hydrolysis with Hydrochloric Acid—Following the isolation of *l*-thyroxine subsequent to hydrolysis with the sulfuric acid-butyl alcohol mixture it was of interest to determine whether similar results could be obtained by the use of hydrochloric acid. For this purpose 600 gm. of iodinated casein from the same lot used for the previous work were treated exactly as already described except that 20 per cent hydrochloric acid was used for the hydrolysis instead of sulfuric acid. After the material was refluxed for 10 hours, the biuret test became negative and hydrolysis was judged to be complete. The hydrolysate differed from that obtained with sulfuric acid in the much larger amount of insoluble residue that collected below the butyl alcohol layer during the extraction with sodium hydroxide. This was drawn off through the separatory funnel after the final extraction and discarded. After removal of the butyl alcohol, and purification as described previously, a yield of 31 mg. of a crystalline product containing 64.2 per cent iodine and of the same appearance and general properties as thyroxine was isolated. In view of the results described previously, it appeared safe to assume that this compound was also *l*-thyroxine. Because of the small amount of material available, however, further identification was not attempted.

Biological Assay of l-Thyroxine—The metabolic effect of *l*-thyroxine obtained by the hydrolysis of iodinated casein with sulfuric acid was compared with that of crystalline synthetic *dl*-thyroxine by use of the guinea pig assay described in the preceding paper. Both preparations were injected subcutaneously.

A stock solution of *l*-thyroxine in a concentration of 1 mg. per ml. was prepared. Before being made up to volume, the thyroxine was dissolved by adding a minimum amount of 0.1 N sodium hydroxide, and then precipitated as the monosodium salt by addition of 0.1 N hydrochloric acid to pH 6.8 to 7.0. Immediately before injection the stock solution was mixed well and an aliquot was accurately pipetted out. 4 drops of 0.1 N sodium

hydroxide solution were added to dissolve the thyroxine, and the solution was made up to the required volume. The *dl*-thyroxine was dissolved in distilled water with the aid of a minimum of 0.1 N sodium hydroxide solution. The solutions were kept in the refrigerator when not in use.

The results of two different assays in which the calorigenic effect of the *l*- and *dl*-thyroxine is compared are given in Table I. In the first, *l*-thyroxine from iodinated casein, injected at the rates of 4 and 8 γ per 100

TABLE I

Comparative Thyroidal Potency of l- and dl-Thyroxine

Preparation	Dosage	No. of guinea pigs	Average gain in CO_2 output*					Body weight loss	
			Trial 1	Trial 2	Trial 3	Trial 4	Combined average	After 7 days dosage	After 14 days dosage
Assay 1									
	γ per 100 gm		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
l-Thyroxine, from iodocasein	8	4	53.1	52.7	49.6	46.1	50.4	12.0	20.2
" "	4	4	29.4	25.1	40.6	39.0	33.5	9.5	14.6
dl-Thyroxine, synthetic	8	4	32.4	26.5	37.1	36.0	33.0	8.6	14.0
Assay 2									
l-Thyroxine, from iodocasein	6.25	4	29.6	33.6	32.7	33.4	32.3	10.6	18.3
" "	3.12	4	25.5	22.9	25.5	20.9	23.7	6.4	10.7
dl-Thyroxine, synthetic	6.25	4	24.8	27.7	27.7	24.0	26.0	7.2	11.4

* The metabolism data for Assay 1 are for the 9th, 10th, 11th, and 14th days after dosage started. The values for Assay 2 were obtained on the 7th, 8th, 9th, and 12th days of dosage. The results are expressed as the percentage increase in carbon dioxide output above normal control values obtained under similar conditions.

gm. of body weight, was compared with crystalline synthetic *dl*-thyroxine injected at the 8 γ level.

In Assay 1 the carbon dioxide production was determined periodically during the 6th to 14th days of dosage. The values obtained in four trials during the latter part of the period, when the animals had reached the peak of stimulation, are presented in Table I. The percentage loss of body weight was calculated on the 7th and 14th days of dosage. Both the increase in carbon dioxide production and loss of body weight of the animals receiving 4 γ of *l*-thyroxine or 8 γ of *dl*-thyroxine per 100 gm. of body

weight were practically identical. 8 γ of *l*-thyroxine produced a much higher response than the same dosage of the *dl* mixture.

The results of a second trial in which *l*- and *dl*-thyroxine were compared at a lower dosage level are shown in Assay 2, Table I. After 12 days of dosage on this experiment, there was a sudden rise in environmental temperature that made it impossible, under our conditions, to obtain reliable results on metabolism. Therefore, results on the carbon dioxide output are given for the last four trials in this 12 day period, and data on loss of body weight are given for the 7th and 14th days of dosage. In this instance

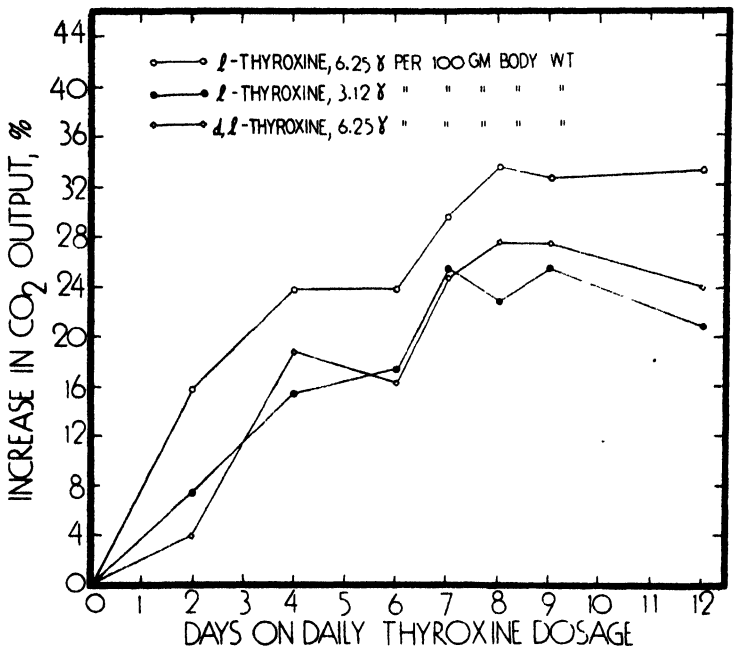


FIG. 1. Comparative metabolic effect of *l*- and *dl*-thyroxine

6.25 γ of *dl*-thyroxine appeared to cause a slightly greater effect than 3.12 γ of *l*-thyroxine, the two substances producing average increases in carbon dioxide output of 26.0 and 23.7 per cent, respectively. From data that have accumulated in our laboratory (unpublished), we have computed that the standard error ($5y \times x$) of the increased carbon dioxide output in response to a given dosage of thyroxine is 3.86 per cent. Therefore, the difference noted above is within the experimental error of the method. When given at the same dosage level, *l*-thyroxine again produced a distinctly higher response than the *dl* compound did.

This is clearly demonstrated by the response curves of Assay 2, charted

in Fig. 1. It will be noted that the responses to 3.12 γ of *l*-thyroxine and 6.25 γ of *dl*-thyroxine per 100 gm. of body weight run closely parallel but are distinctly lower than the values for the group receiving 6.25 γ of *l*-thyroxine.

DISCUSSION

From the results, it must be concluded that the *l*-thyroxine obtained by acid hydrolysis of iodinated casein has approximately twice the potency of synthetic *dl*-thyroxine. This is in agreement with the report of Foster *et al.* (2), who reported that *l*-thyroxine recovered from thyroid tissue exerted twice the effect of the *dl* mixture. Gaddum (8) reported that *l*-thyroxine obtained by resolution of the synthetic substance had 3 times the potency of the *d* form, while Salter, Lerman, and Means (9) failed to establish any difference between these same preparations when administered to myxedematous patients. Foster *et al.* suggested that the small amount of activity noted by Gaddum for *d*-thyroxine may have been due to incomplete resolution of the sample, which possessed a somewhat lower specific rotation than the natural product used in their experiments. From our present results it appears possible to account, within experimental error, for the full activity of *dl*-thyroxine by the activity of its *l* component alone. Since the accuracy of the method is limited to approximately ± 20 per cent, however, a small amount of activity of the *d* compound could exist without being detected.

The present results help to throw some additional light on the mode of synthesis of thyroxine in an iodinated protein. If the synthesis occurs by the oxidative coupling of 2 molecules of diiodotyrosine and the elimination of one side chain, as has been previously suggested by Ludwig and von Mutzenbecher (7), Harington and Rivers (10), Reineke *et al.* (11), and elaborated further by Johnson and Tewkesbury (12), the thyroxine would be expected to occur in an optically active state, since no alteration of the remaining side chain would be involved. Our recovery of the *l* compound after acid hydrolysis lends further support to this concept.

The formation of *l*-thyroxine in iodinated proteins also explains in large part the high thyroidal activity of the preparations previously reported from our laboratory, as discussed in the preceding paper.

By our new method of hydrolysis a yield of approximately 0.1 per cent of *l*-thyroxine was obtained as compared to more than 0.4 per cent of *dl*-thyroxine obtained by hydrolysis with alkali. It is believed that, by further study of the method of hydrolysis with acid in order to establish more nearly optimum conditions, this yield could be improved considerably. Even as used at present, however, it represents a great advance over any method for obtaining *l*-thyroxine that has been available heretofore. Harington and Salter (1) obtained minute yields of *l*-thyroxine from thyroid

by enzymatic hydrolysis. Foster *et al.* (2), using similar methods, recovered a yield of only 104 mg. of *l*-thyroxine from 18 kilos of fresh hog thyroid. In a second trial they reported a yield of 28 mg. of *l*-thyroxine from 2.2 kilos of commercial desiccated and defatted thyroid.

SUMMARY

1. Crystalline *l*-thyroxine ($[\alpha]_D = -4.2°$, iodine content 65.1 per cent, m.p. 236–238°) was isolated from iodinated casein.

2. Direct hydrolysis of iodinated casein with a sulfuric acid-butyl alcohol mixture gave a yield of approximately 0.1 per cent of crystalline *l*-thyroxine. Thyroxine was recovered in a much lower yield by direct hydrolysis with a hydrochloric acid-butyl alcohol mixture.

3. Details of the new method for recovery of thyroxine are described.

4. The *l*-thyroxine showed approximately twice the thyroidal effect of racemic thyroxine, as shown by its elevation of the carbon dioxide output and by loss of body weight of guinea pigs.

BIBLIOGRAPHY

1. Harington, C. R., and Salter, W. T., *Biochem. J.*, **24**, 456 (1930).
2. Foster, G. L., Palmer, W. W., and Leland, J. P., *J. Biol. Chem.*, **115**, 467 (1936).
3. Baumann, C., *Munch. med. Woch.*, **43**, 476 (1896).
4. Kendall, E C., Thyroxine, New York (1929).
5. Lerman, J., and Salter, W. T., *Endocrinology*, **25**, 712 (1939).
6. Lerman, J., and Salter, W. T., *Endocrinology*, **18**, 317 (1933).
7. Ludwig, W., and von Mutzenbecher, P., *Z. physiol. Chem.*, **258**, 195 (1939).
8. Gaddum, J. H., *J. Physiol.*, **68**, 383 (1929–30).
9. Salter, W. T., Lerman, J., and Means, J. H., *J. Clin. Inv.*, **14**, 37 (1935).
10. Harington, C. R., and Rivers, R. V. P , *Nature*, **144**, 205 (1939).
11. Reineke, E. P., Williamson, M. B., and Turner, C. W., *J. Biol. Chem.*, **143**, 285 (1942).
12. Johnson, T. B., and Tewkesbury, L. B., Jr., *Proc. Nat. Acad. Sc.*, **28**, 73 (1942).

LETTERS TO THE EDITORS

ANOMALOUS AMINO NITROGEN VALUES*

Sirs:

As a preliminary step in the investigation of the structure of penicillin, amino and total nitrogen determinations were made on a crude preparation.[1] It was found that the manometric amino nitrogen value was almost double that for total nitrogen (micro-Kjeldahl). Chrysogenin,[1] the yellow pigment which accompanies penicillin, gave an amino nitrogen value of 2.73 per cent (30 minutes reaction time), although the micro-Kjeldahl determination showed that the material contained no nitrogen. It is, of course, well known that certain amino acids give more than the theoretical amount of nitrogen in the Van Slyke determination.[2] However, there are very few reports concerning anomalous values from non-nitrogenous compounds. Lough and Lewis[3] found that thioglycolic and α-thiolpropionic acids yielded nitrogen. Clarke and Inouye[4] observed that pyruvic acid gave an appreciable amount of gas when analyzed by the standard procedure. It seemed desirable, therefore, to determine what types of non-nitrogenous compounds give amino nitrogen values. Some preliminary results of this study are recorded in the table. The determinations were made by the standard Van Slyke manometric technique at temperatures of 24–28°.

These results clearly demonstrate that amino nitrogen values on compounds of unknown structure must be interpreted with caution. In the case of the crude penicillin, for example, the amino nitrogen figure by itself is quite misleading, since the available evidence indicates that penicillin does not contain a free primary amino group.

The identity of the gas produced in the reaction of nitrous acid with resorcinol was of interest in connection with the mechanism of the reaction. The possibilities are nitrogen, carbon monoxide, hydrogen, or a hydrocarbon. Carbon monoxide was eliminated, since the gas was not absorbed by Winkler's solution. It is highly improbable that either hydrogen or a

* The authors wish to acknowledge a grant to one of us (S. R. D.) by The Upjohn Company.

[1] This product was kindly supplied by The Upjohn Company.

[2] Schmidt, C. L. A., *J. Biol. Chem.*, **82**, 587 (1929). Kendrick, A. B., and Hanke M. E., *J. Biol. Chem.*, **117**, 161 (1937).

[3] Lough, S. A., and Lewis, H. B., *J. Biol. Chem.*, **104**, 601 (1934).

[4] Clarke, H. T., and Inouye, J. M., *J. Biol. Chem.*, **89**, 399 (1930).

Compound	Reaction time	Amino nitrogen	
		per cent	*atoms per mole sample*
Resorcinol	5	9.44	0.75
	30	12.91	1.03
" monomethyl ether	5	1.10	
" dimethyl ether	5	0.05	
Catechol	5	3.87	0.30
	30	7.62	0.58
Hydroquinone	5	3.63	0.28
	30	4.63	0.36
Salicylic acid	30	0.21	
m-Hydroxybenzoic acid	5	0.59	
p-Hydroxybenzoic "	5	0.76	
Phenol	5	1.00	
Ascorbic acid	5	0.00	
	30	0.06	
Ethyl acetoacetate	30	0.24	
Tetronic acid	30	2.08	

hydrocarbon is produced in this reaction. The most likely possibility is that nitrogen is formed by reduction of the nitrous acid of the reagent. A more extensive investigation of this question is now in progress.

Division of Biochemistry
Noyes Laboratory of Chemistry
University of Illinois
Urbana

HERBERT E. CARTER
S. R. DICKMAN

Received for publication, May 17, 1943

SOME PROPERTIES OF A GROWTH FACTOR FOR LACTOBACILLUS CASEI*

Sirs:

This communication describes certain properties of a growth factor for *Lactobacillus casei* ϵ, first described as the "norite eluate factor."[1] Preparations have been obtained from liver and yeast. The compound from liver gave a methyl ester which was precipitated repeatedly from ethanol in a yellow gelatinous form without change in biological activity. Analysis, C 52.7, H 4.8, N 20.1, phosphorus absent.[2] Upon slow evaporation on a microscope slide in air of a methanol solution of the methyl ester, very small acicular or bladed crystals, or aggregates of these, were formed which showed oblique extinction. The ultraviolet absorption spectrum of the free acid was measured in acid, neutral, and alkaline solutions (Fig. 1). From yeast a compound was obtained which yielded a methyl ester in crystalline form. The free acid of this had the same absorption spectrum in 0.1 N NaOH, pH 7.0, as the material isolated from liver. The esters obtained from liver and yeast were hydrolyzed to yield preparations which had equal potency for *L. casei* ϵ. However, when assayed with *S. lactis* R, by the method of Mitchell and Snell,[3] the preparation from yeast was only half as active as that from liver. When compared with Liver Fraction B (potency 1) of Mitchell and Snell,[3] our preparation from liver had a relative potency of 79,000 when determined with *L. casei* ϵ and 78,000 when assayed with *S. lactis* R. The preparation from yeast had a relative potency of 75,000 with *L. casei* ϵ and 38,000 with *S. lactis* R. These results indicate that the materials isolated from liver and yeast are different.

The compound obtained from liver is thought to be identical with that obtained by Pfiffner *et al.*,[4] who reported an analysis for the free acid which is in agreement with the present results with the ester if these are adjusted for an added methyl group. The amount required for half maximum growth of *L. casei* ϵ was 0.000055 γ per ml. of medium, which is in good

* The author wishes to thank Miss B. Eames for making the microbiological assays, Mr. D. Richardson for the absorption spectrum measurements, and Dr. E. F. Williams for the crystallographic work.

[1] Snell, E. E., and Peterson, W. H., *J. Bact.*, **39**, 273 (1940). Hutchings, B. L. Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

[2] The writer wishes to take the opportunity to correct an error in a previous report (*J. Biol. Chem.*, **139**, 475 (1941)) that the growth factor for *L. casei* ϵ contains phosphorus.

[3] Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub., No. 4137*, 36 (1941).

[4] Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404 (1943).

agreement with the results obtained by Pfiffner *et al.*[4] Both the preparations from liver and yeast appear to differ from the growth factor for *S. lactis* R described by Keresztesy *et al.*[5] Their preparation was approxi-

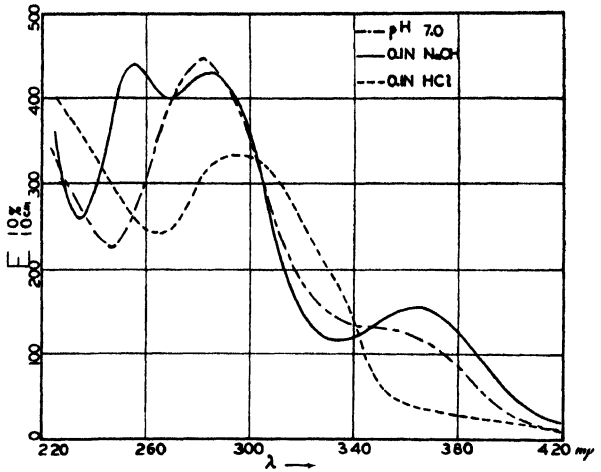


FIG. 1. Ultraviolet absorption spectrum of *L. casei* factor from liver

mately 2500 times as active for *S. lactis* R as for *L. casei* ϵ, while the two preparations described here were more active for *L. casei* than for *S. lactis* R.

Lederle Laboratories, Inc.
Pearl River, New York

E. L. R. STOKSTAD

Received for publication, June 14 1943

[5] Keresztesy, J. C., Rickes, E. R., and Stokes, J. L., *Science*, **97**, 465 (1943).

GROWTH-PROMOTING ACTIVITY OF BETAINE IN THE CHICK

Sirs:

The ineffectiveness of betaine[1] and methionine[2] in the prevention of perosis due to choline deficiency indicates little or no choline synthesis in the chick. Betaine has previously shown small growth-promoting effects of doubtful significance.[1, 3] We wish to report results of studies which show that under certain conditions betaine is capable of exerting a growth effect in the chick equivalent to that of choline. Typical results which have been confirmed by other experiments are given in the table.

The chick diet used was a synthetic type containing isolated soy bean protein as the only source of amino acids. It was otherwise similar to

Effects of Choline, Betaine, and Methionine on Rate of Gain in Chicks

Supplement to diet	Level	Weight gain per day	
		Series I	Series II
	per cent	*per cent*	*per cent*
None		1.9, 2.1*	1.8
Betaine hydrochloride	0.22	3.4	4.1
	0.30	3.7	4.0
Choline chloride	0.20	3.8, 4.2*	3.4, 4.2*
Methionine	0.50	4.4	

* Each value represents one group.

diets already described[4] with the exception that yeast extract was replaced by crystalline members of the vitamin B complex and choline was not added. The diet, in contrast with those previously employed,[1–3] contained amounts of the sulfur-bearing amino acids which were known to be sub-optimal. This fact, together with the deficiency of choline, is believed to have accentuated the effect of betaine. Chicks were depleted of choline prior to their use in experiments.

These and other data obtained indicate that betaine may assume in certain respects the functions of choline or methionine in the chick.

[1] Jukes, T. H., and Welch, A. D., *J. Biol. Chem.*, **146**, 19 (1942). Almquist, H. J., unpublished data (1943).

[2] Jukes, T. H., *J. Nutr.*, **22**, 315 (1941).

[3] McGinnis, J., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol and Med.*, **51**, 293 (1942).

[4] Almquist, H. J., Mecchi, E., Kratzer, F. H., and Grau, C. R., *J. Nutr.*, **24**, 385 (1942).

Betaine or a substance derived from betaine may be a physiological requirement which can be formed, if necessary, from choline or methionine.

Division of Poultry Husbandry
College of Agriculture
University of California
Berkeley

H. J. ALMQUIST
C. R. GRAU

Received for publication, July 3, 1943

α-KETOGLUTARIC DEHYDROGENASE OF HEART EXTRACTS*

Sirs:

The reaction

$$\alpha\text{-Ketoglutarate} + 0.5O_2 = \text{succinate} + CO_2 \qquad (1)$$

was studied in dialyzed extracts of washed cat heart in order to obtain some information on the components of the system, a knowledge which will be of help in the purification of the enzyme or enzymes involved.

In the presence of 0.025 M malonate, to inhibit the succinic dehydrogenase, one obtains R.Q. values of 1.7 to 1.9, and the ratio of molecules of oxygen consumed to molecules of α-ketoglutarate utilized is close to 0.5; this indicates the almost exclusive occurrence of reaction (1) under the experimental conditions employed. The pH optimum of the reaction is about 7.5. After 4 to 5 hours of dialysis there is but little utilization of α-ketoglutarate; this can, however, be restored to values approaching those obtained before dialysis by addition of (*a*) inorganic phosphate (but not arsenate) and (*b*) muscle adenylic acid or adenosine triphosphate (but not yeast adenylic acid or adenosine). Some of these facts are illustrated in the accompanying table. Addition of fumarate has no effect on the rate of α-ketoglutarate utilization, indicating that C_4 dicarboxylic acids are not involved in the transport of hydrogen from α-ketoglutarate to oxygen.

Indirect measurements have shown that oxidation of pyruvate by tissue extracts can be linked with as high an uptake of phosphate by phosphate acceptors as 3 molecules per atom of oxygen consumed.[1] It is therefore of some interest that, in relation to the oxygen consumption, as much esterification of phosphate with glucose is brought about by reaction (1) as by the complete oxidation of pyruvate with the same enzyme preparation. In an experiment with α-ketoglutarate, 16 micromoles of oxygen were consumed, 30 micromoles of CO_2 produced, 40 micromoles of α-ketoglutarate utilized, and 35 micromoles of phosphate esterified with glucose in 40 minutes at 36.6°. This gives an R.Q. of 1.87, a ratio of O_2 consumed to α-ketoglutarate utilized of 0.4, and a P:O ratio of 1.1 molecules of phosphate per atom of oxygen consumed. With pyruvate as substrate (no malonate), 22 micromoles of oxygen were consumed, 12 micromoles of pyruvate utilized, and 52 micromoles of phosphate esterified in 20 minutes

* Supported by a grant from the Williams-Waterman Fund of the Research Corporation.

[1] Ochoa, S., *Federation Proc.*, **2**, 67 (1943).

1.5 cc. of dialyzed extract were brought to 2.0 cc. with additions including 8 micromoles of $MgCl_2$ and 50 micromoles of sodium malonate. Incubation 40 minutes at 36.6°; 100 per cent oxygen in the gas phase. All values are expressed in micromoles.

	Additions				Oxygen uptake	α-Keto-glutarate utilized
	α-Keto-glutarate	Phosphate	Arsenate	Adenine nucleotide		
1	0	75	0	5 AMP	0.1	
	56	75	0	0	1.1	3.4
	56	75	0	5 AMP	8.3	17.8
	56	0	0	5 "	1.1	4.0
	56	0	75	0	1.9	7.1
	56	0	75	5 AMP	1.2	4.4
2	65	75	0	0	1.3	10.0
	65	75	0	1.35 ATP	19.6	38.0

AMP = adenosine-5-monophosphate; ATP = adenosine triphosphate.

(O_2 consumed to pyruvate utilized = 1.8; P:O = 1.2). In another experiment the P:O ratios were 1.5 with α-ketoglutarate, and 1.7 with pyruvate.[2]

Department of Medicine SEVERO OCHOA
New York University College of Medicine and the Medical Service of the Psychiatric Division, Bellevue Hospital
New York

Received for publication, July 13, 1943

[2] The values of the P:O ratio as directly measured depend on the activity of the adenosine triphosphatase in the extracts, varying inversely with it.

INDEX TO AUTHORS

L

M

O

P

INDEX TO SUBJECTS

D

E

F